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THE METABOLISM AFTER MEAT FEEDING OF DOGS IN WHICH PANCREATIC EXTERNAL SECRETION WAS ABSENT.

BY FRANCIS G. BENEDICT AND JOSEPH H. PRATT.

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Boston, Mass., and the Laboratory of the Theory and Practice of
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(Received for publication, April 7, 1913.)

The well-known increase in the total metabolism of both animals and man resulting from the ingestion of various kinds of food has been the subject of much study, and while investigators are well in accord as to there being such an increase, they differ widely in their opinion as to the cause. At present there are two distinctly different theories. Unfortunately neither of these is sufficiently clarified to be sharply defined, and as experimental evidence accumulates there seems to be more or less of a tendency for the two theories to coalesce. In general it may be stated that the theory most actively championed by Zuntz and his co-workers assumes that the increase in metabolism is mainly due to the mechanical processes of digestion, including the work of peristalsis, segmentation, absorption, and glandular activity. Rubner, on the other hand, holds that this increase is due to the specific dynamic action of the foodstuffs, *i.e.*, a quota of heat production that appears as free heat and does not benefit the cells. It is thus seen that in one case the increase in metabolism is considered to be due chiefly to mechanical causes, while in the other it is mainly attributed to chemical processes.

Experimental evidence has accumulated to such an extent that we may be certain that an increased metabolic activity follows the ingestion of all of the three main nutrients—protein, fat, and carbohydrate. This is greatest in the case of protein; in fact, so

¹ This investigation was aided by a grant from the Proctor Fund for the Study of Chronic Diseases.

great is the increase in metabolism following the ingestion of protein that Zuntz himself is inclined to maintain that the work of digestion alone is not sufficient to account for this increased activity, and to accept the view that there is also a chemical action.² Rubner, on the other hand, is apparently unwilling to admit that the mechanical work of digestion plays any rôle.

A discussion of the general fundamental question of the specific dynamic action of foodstuffs and of the work of digestion is not appropriate to this paper, and is therefore deferred until the appearance of the results of a large amount of experimental work on man which is now being prepared for publication by Mr. T. M. Carpenter of the Nutrition Laboratory. An opportunity offered, however, for making a study of the metabolic changes following the ingestion of meat upon three dogs without external pancreatic secretion, whose power to digest and absorb protein was greatly diminished. While in recent years considerable evidence has accumulated as a result of surgical interference with the normal digestive tract, it has usually been obtained when the dogs were under the influence of curare or some narcotic, and the question has rightly been raised as to whether the results obtained under such conditions can be in any way normal.³ Our three dogs, however, made an excellent recovery and continued in good health after the pancreatic secretion had been entirely excluded from the intestines; their absorption of protein and fat was then studied. Since absorption experiments with these dogs showed that a large amount of undigested meat was lost in the feces, it was evident that under these circumstances there would be a maximum work of digestion so far as peristalsis and segmentation were concerned, and a decreased absorption of material from the foodstuffs to be carried by the blood to the cells, there, according to Rubner's views, to produce, in part, free unutilized heat. It was believed, therefore, that a study of the metabolism of these animals to determine the effect of ingesting meat, if made with the proper technique, would throw light upon the general question of the cause of the increased metabolism following the ingestion of food.

² Zuntz and Loewy: *Lehrbuch der Physiologie des Menschen*, Leipzig, 1909, p. 678. See also, Loewy: *Oppenheimer's Handbuch der Biochemie*, Jena, 1911, iv, p. 271.

³ Zuntz: *Oppenheimer's Handbuch der Biochemie*, Jena, 1911, iv, p. 855.

Review of earlier work on the effect of the exclusion of the pancreatic secretion upon the absorption of food.

Abelmann,⁴ working under the direction of Minkowski, found that after the extirpation of the pancreas in the dog there was a great decrease in the absorption of nitrogen and fat; in fact, when meat was fed, there was no absorption of fat. After total extirpation of the pancreas, the percentage of nitrogen absorbed varied from 27.8 to 58 per cent. When pancreas was added to the diet, the absorption rose to 78 per cent. After partial extirpation of the pancreas, the absorption of nitrogen varied from 40 to 83 per cent. Unfortunately most of the experiments were of only one day's duration.

Rosenberg⁵ made absorption studies on dogs in which he had separated the pancreas from the intestine. The absorption of nitrogen varied in experiments in which a meat diet was given from 64.1 to 83 per cent, while normally it was above 90 per cent. Lombroso⁶ and also Zunz and Mayer⁷ concluded from their experiments that all of the pancreatic juice could be excluded from the intestine without serious disturbance in the absorption of proteins and fats. These investigators stated that they tied "both" pancreatic ducts, but Hess⁸ and his student Sinn⁹ have shown that the pancreas in the dog has usually three ducts and sometimes four. In eight experiments on dogs, Hess¹⁰ succeeded only twice in tying all the ducts at operation and these two animals were the only ones in his series in which the absorption of fats and proteins was definitely reduced. With one of these dogs, 54.68 per cent of nitrogen was absorbed, and with the other, 42 per cent.

Niemann,¹¹ working under the direction of Brugsch, also studied the absorption in dogs after tying the pancreatic ducts. Although the claim was made that no pancreatic juice entered the intestine, one dog was found to absorb 93.9 per cent of nitrogen and another dog 92.9 per cent. A meat diet was used in both experiments. Fleckseder,¹² investigating the same problem in Hans Meyer's laboratory, concluded that normal absorption can occur even when all the pancreatic juice is excluded from the intestine.

The observations published by Pratt, Lamson, and Marks¹³ in 1909

⁴ Abelmann: Inaugural Dissertation, Dorpat, 1890.

⁵ Rosenberg: *Pflüger's Archiv*, lxx, p. 371, 1898.

⁶ Lombroso: *Pflüger's Archiv* cxii, p. 531, 1906; *Arch. f. exp. Path. u. Pharm.*, lviii, p. 251, 1908.

⁷ Zunz and Mayer: *Mém. couronnées et autres mém. p. p. l'Acad. Royale de Méd. de Belgique*, xviii, p. 68, 1904.

⁸ Hess: *Pflüger's Archiv*, cxviii, p. 536, 1907.

⁹ Sinn: Inaugural Dissertation, Marburg, 1907.

¹⁰ Hess: *Medizin.-Naturwiss. Archiv*, i, p. 161, 1908.

¹¹ Niemann: *Zeitschr. f. exp. Path. u. Ther.*, v. p. 456, 1909.

¹² Fleckseder: *Arch. f. exp. Path. u. Pharm.*, lix, p. 407, 1908.

¹³ Pratt, Lamson, and Marks: *Trans. of the Assoc. of Amer. Physicians*, xxiv, p. 266, 1909.

showed that the good absorption obtained by the earlier investigators was due to failure in excluding permanently the pancreatic secretion from the intestine. In experiments upon a series of four dogs, in which the pancreas had been separated from the duodenum, marked diminution in the absorption of fat and nitrogen was found. The absorption of nitrogen ranged from 22.2 to 61.7 per cent. In all of these animals, great atrophy and sclerosis of the pancreas occurred. The operations were made by Dr. Fred T. Murphy and in only one of five dogs did he fail to shut out the pancreatic juice from the intestine. In this animal, which was the first of the series, there was no decrease in absorption of either the protein or the fat. At the autopsy the pancreas was of normal size and consistence and a sinus was found connecting the interior of the duodenum with the main duct of the pancreas.

Description of the operations on the dogs used in this research.

Zep. One of the three dogs used in the present study, "Zep," was a female which, while a puppy and weighing 5.7 kgms., had had the pancreas separated from the duodenum. This was more than two and a half years prior to the date of our experiments in the respiration apparatus, the operation being performed by Dr. F. T. Murphy on November 19, 1908. Ether was used as the anaesthetic, and a linear incision was made in the middle line of the abdomen. The pancreas was about 10 cm. long. Two ducts were found and cut between double ligatures. All the connections between the pancreas and duodenum were severed except the main branches of the arteria and vena pancreatico-duodenalis. A portion of the omentum was then placed about the duodenum, so that the corpus pancreatis¹⁴ and duodenum were not in contact at any point.

Pat. This dog was a medium-sized, healthy male, weighing 11 kgms. The operation was performed February 13, 1911. Prior to anaesthesia by ether, 3 cc. of 1 per cent solution of morphia sulphate were injected. On opening the abdominal cavity by the usual incision, the internal end of the spleen was found to be almost in the median line. The terminal portion of the processus uncinatus of the pancreas, which measured 5 by 3 cm., was separated from the gland by cutting between double ligatures of black silk. An incision was also made in the lower portion of the internal surface of the spleen; a large pocket was then made and the portion

¹⁴ For nomenclature, see *Pflüger's Archiv*, cxviii, p. 267, 1907.

of the processus uncinatus separated from the remainder of the gland was placed in this. The vascular stalk, which entered the extreme end of the processus uncinatus and which contained an artery and vein, both of goodly size, was preserved. There was some difficulty in introducing the pancreatic tissue into the pocket made in the spleen, and during this manipulation considerable blood was lost as there was constant oozing from the cut tissues of the spleen. Finally, all the pancreatic tissue, except about 1 cm. of the terminal portion, was within the splenic substance. The graft was secured in place by sutures and the omentum was drawn around the vascular stalk. The dog made a good recovery.

A second operation was performed by Dr. Murphy on March 7, 1911. An injection of morphine was given before etherization, the dog vomiting within ten minutes. Very little ether was required. A thin linear scar marked the site of the old operation wound. When the abdomen was opened, a large mass was found behind the coils of intestine with its anterior surface covered with mesentery. After careful dissection this tumor was found to be an abscess occupying the lower end of the spleen which had been drawn forward. This was opened and there escaped about 30 cc. of thin, pinkish, puriform material with a pleasant odor resembling that of heliotrope. There were many adhesions of mesentery to the internal surface of the spleen, and a nodule of pancreatic tissue about 2 cm. in size projected from the splenic tissue. There was no evidence of inflammation about the duodenum or pancreas. The mesenteric stalk which carried the blood vessels to the graft in the spleen was cut. The duodenum was stripped clean of pancreatic tissue and two ducts were found and cut between double ligatures; the pancreatoco-duodenal artery and vein were spared. The body of the pancreas and the splenic process were then quickly extirpated, the weight of the pancreatic tissue being 19 grams.

Flora. The dog "Flora" was an old, fat, female dog, weighing 13.8 kgms. The operation was performed on March 29, 1912, by Dr. Beth Vincent. Under ether narcosis, the corpus pancreatis was carefully separated from the duodenum; the pancreatic ducts were cut between double ligatures, the main branches of the pancreatoco-duodenal artery and vein being preserved. Strong ligatures were placed about the gland at the junction of the corpus with the processus lienalis above and at the boundary of the corpus

and the processus uncinatus below; the gland was then sectioned proximally to each of these ligatures and the corpus pancreatis removed. The excised tissue weighed 10 grams. Omentum was placed over the stumps of the processus lienalis and processus uncinatus.

Methods for preparation and analysis of foods and feces.

During the absorption experiments, the dogs were confined in metabolism cages. The diet proposed by Gies¹⁵ was used in most of the experiments. This consisted of a mixture composed of 250 grams of chopped meat, 70 grams of cracker meal, 50 grams of bone ash, and 30 grams of lard. For demarcating the stools, carmine and charcoal were employed, but in the absorption experiment with "Flora" in which an exclusive meat diet was used, demarcation was obtained by feeding milk and bread, thus producing bright yellow feces which contrasted sharply with the dark meat stools. The method for determining the composition of the food was as follows: An aliquot part, as a fifth or a tenth, of the amount of each article of diet eaten daily by the dog was taken and placed in a large evaporating dish on the water bath. The composite mixture was analyzed as a whole. For the nitrogen determinations the Kjeldahl method was employed, a determination of the nitrogen in the meat used (finely chopped beef heart) being made for each experiment with the respiration apparatus.

Absorption experiments with the dog "Zep."

Nine absorption experiments were made with the dog "Zep." The details of the first experiment are as follows:

First absorption experiment. November 27-30, 1908; duration, 4 days; weight of dog November 27, 5.3 kgms.

Total food: 500 grams chopped beef heart; 100 grams bone ash; 140 grams cracker meal; 60 grams lard.

Weight of dried food, 425 grams; nitrogen in food, 4.59 per cent; amount of nitrogen, 19.5 grams.

Weight of dried feces, 265.5 grams; nitrogen in feces, 4.99 per cent; amount of nitrogen, 13.25 grams; nitrogen absorbed, 32.1 per cent; nitrogen lost in feces, 67.9 per cent.

¹⁵ Gies: *Amer. Journ. of Physiol.*, x, p. 22, 1904.

A summary of the results obtained in all of the absorption experiments with Zep is given in table 1. The absorption of nitrogen varied from 32.06 per cent to 60.20 per cent unless pancreas or pancreatic preparations were added to the diet.

The details of the absorption experiments with the dogs "Pat" and "Flora" are as follows:

Absorption experiment with the dog "Pat."

Date, March 20-22, 1911; duration, 3 days; weight of dog March 20, 8.125 kgms.

Total food: 750 grams chopped beef heart; 210 grams cracker meal; 120 grams bone ash; 60 grams lard

Weight of dried food, 438 grams; nitrogen in food, 4.73 per cent; amount of nitrogen, 20.78 grams.

Weight of dried feces, 342 grams; nitrogen in feces, 4.41 per cent; amount of nitrogen in feces, 15.08 grams; nitrogen absorbed, 27.92 per cent; nitrogen lost in feces, 72.08 per cent.

Absorption experiment with the dog "Flora."

Date, November 12-15, 1912; duration, 4 days; weight of dog November 12, 6.6 kgms.

Total food: 3000 grams chopped beef heart.

Weight of dried food, 767 grams; nitrogen in food, 10.87 per cent; amount of nitrogen, 83.31 grams.

Weight of dried feces, 395.3 grams; nitrogen in feces, 8.90 per cent; amount of nitrogen, 35.18 grams; nitrogen absorbed, 57.77 per cent; nitrogen lost in feces, 42.23 per cent.

Measurements of the metabolism.

Unquestionably the ideal measurements of the metabolism of the living organism include the determinations of the carbon-dioxide output, the oxygen intake, the heat production, and the usual analyses of food, feces, and urine. Such complete experiments are extremely expensive and time-consuming, so that they are justifiable only after preliminary experiments in which the factors affecting metabolism have been so carefully studied and controlled as to insure that the elaborate records for the oxygen determinations and the heat measurements will give results not vitiated by extraneous, uncontrolled factors. To be of any value, the determinations of the oxygen consumption must be accurate

TABLE 1.

Summary of results obtained in absorption experiments with the dog "Zep."*

EXPERIMENT NO.	DATE	DURATION	BODY WEIGHT	NITROGEN IN FOOD	NITROGEN IN FECES	TOTAL NITROGEN LOST IN FECES	DIET
		days	kilos	grams	grams	per cent	
1	1908 Nov. 27-30	4	5.3	19.5	13.3	67.9	{ 500 gms. chopped beef heart 100 gms. bone ash 140 gms. cracker meal 60 gms. lard
2	1909 Feb. 2-5	4	5.5	78.9	31.4	39.8	{ 1706 gms. chopped beef heart 341 gms. bone ash 478 gms. cracker meal 80 gms. lard
3	Apr. 4-7	4	6.25	44.1	25.4	57.6	{ 1000 gms. chopped beef heart 200 gms. bone ash 280 gms. cracker meal 80 gms. lard
4	Apr. 12-15	4	6.25	42.3	18.6	44.0	{ 1000 gms. chopped beef heart 200 gms. bone ash 280 gms. cracker meal 1.8 gms. of "holadin," a pancreatic preparation, given daily
5	Oct. 12-15	4	6.45	193.3	109.8	57.1	{ 4000 gms. chopped beef heart 1050 gms. bone ash 100 gms. crushed bone 1200 gms. cracker meal 600 gms. lard
6	Dec. 1-5	5	6.3	21.9	10.0	45.5	3800 cc. milk
7	Dec. 7-12	6	6.1	36.3	16.4	45.3	6260 cc. milk
8	1910 Feb. 8-11	4	5.1	45.5	7.9	17.2	{ 1000 gms. chopped beef heart 280 gms. cracker meal 80 gms. lard 200 gms. bone ash 200 gms. fresh sheep pancreas
9	1911 May 24-26	3	7.3	84.8	37.2	43.9	{ 3000 gms. chopped beef heart.

* Pancreas separated from duodenum and all pancreatic secretion excluded from intestine.

to within 5 per cent, at least, of the true amount. The same may be said regarding the heat measurements. Obviously, the nearer the results approach to accuracy, the greater their value. Fortunately the carbon-dioxide excretion may be determined with great exactness, so that when the dietetic conditions are constant, as was the case in this research, the errors incidental to the computation of the energy output from the carbon-dioxide production are reduced to a minimum, especially with normal animals.

Respiration apparatus. The carbon dioxide produced by the dogs used in these experiments was determined by means of a slightly modified form of the Benedict and Homans apparatus which has previously been described.¹⁶ In this apparatus the animal remains in a closed chamber—in this instance, with a capacity of 1000 liters¹⁷—from which the air is continually withdrawn by means of a rotary blower. The ventilating current is first passed through sulphuric acid to remove the water vapor, then through soda lime to remove the carbon dioxide, and again through sulphuric acid to remove the small amount of moisture added to the dry air as it passed through the moist soda lime; it is then returned to the chamber. By this process the total amount of carbon dioxide produced is absorbed. As the oxygen of the air is consumed, the deficiency is supplied by admitting a fresh supply from a steel cylinder of compressed oxygen. The chamber itself is of galvanized iron, with a top which rests in a water seal. A rubber diaphragm made from a bathing cap is attached to a pipe at one side of the chamber, thus providing for slight expansions and contractions of the air in the system, and also controlling the admission of oxygen.

In the experiments in this study, the dogs were kept inside the chamber for twenty-four to thirty-six hours, the carbon dioxide being collected and weighed for each 4-hour period as it was possible to change from one set of air purifiers to another every 4 hours. The measurement of the oxygen consumption was attempted in only one series of experiments, later described.

¹⁶ Benedict and Homans: *Journ. of Med. Res.*, xxv. p. 409, 1912.

¹⁷ In certain experiments with a control dog "Clara" and in all the experiments with the dog "Flora" a smaller chamber was used, having a capacity of only 280 liters.

Records of muscular activity. As a result of experience with this apparatus we believe that every properly conducted metabolism experiment should consist of two inseparable parts, first, a record of chemical data for the carbon dioxide produced and, when feasible, for the oxygen consumption and heat production; and second and of equal value, a graphic record of the muscular activity or the degree of quietness of the subject. In no research thus far made in this laboratory have these graphic records of the degree of muscular activity or rest been of such vital importance. The method of obtaining these records, which is very simple, has already been described by Benedict and Homans.¹⁸ One end of the cage in which the dog is confined inside the chamber rests on a knife edge bearing, the other end being supported by a strong spiral spring. The weight of the animal is thus equally divided between the knife edge and the spring. Beside the spring supporting the cage is placed a pneumograph supplied by the Harvard Apparatus Company, consisting of a rubber tube reinforced inside by a brass spiral spring. The elongations of the spiral spring are accompanied by similar elongations of the pneumograph, the confined air inside the rubber tubing expanding or contracting with each movement of the cage. This expansion or contraction of the air inside the pneumograph is transmitted to a rubber tube outside connected with a delicate Marey tambour which records with a pointer on a smoked drum. Every change in the center of gravity of the animal alters the tension of the spring, thus producing a corresponding movement of the pointer. With the small apparatus described by Benedict and Homans, it is possible so to adjust the sensitiveness of the apparatus as to record even the respirations of the dog, inasmuch as the slight change in the center of gravity of the animal during a respiration produces a change in the tension of the spring and a movement of the pointer. Such a degree of refinement was entirely unnecessary in these experiments and was not employed with the larger apparatus used for this study. The minor muscular movements, such as slightly moving the head, one of the legs, or even the tail, could, however, be easily identified, and during the whole progress of the experiment, a kymograph record was made of the movements of the cage.

¹⁸ Benedict and Homans: *loc. cit.*

Importance of kymograph records. In studying the effect of any superimposed factor upon the fasting metabolism it is important to know whether an increase in metabolism, if noted, is due to extraneous muscular activity or to internal activity, but without a record of the external muscular activity, no adequate idea of the transformations in the body can be obtained. In very few of these experiments was there sufficient movement on the part of the dog to be visible to the observer and, in general, we might have stated that in practically all of the experiments the dog was resting quietly in the chamber. By means of the kymograph records, however, it was shown that there were noticeable variations in the muscular activity in the different periods—variations that certainly produced a change in the metabolism not due to the internal activity. The method of selecting periods for comparison was as follows:

The kymograph records were carefully examined by an assistant and from the excursions of the tambour, each period was classified as one of a number of degrees of activity varying from very quiet to moderately active. An independent estimate of the variations in muscular activity was subsequently made by the same person. This duplicate estimate was repeated by another assistant, so that four estimates were made which agreed remarkably well. All periods estimated as active or above were discarded, and only those periods which were considered as quiet were used for comparison. An exact estimate of the relative values for the experimental periods was of course difficult, but it is probable that a reasonably approximate estimation was secured and that the periods selected for comparison are at least fairly representative of the conditions inside the chamber.

In this way and this way only can we be sure of suitable and proper bases for a comparison of the metabolism in the different periods. It would be obviously illogical to compare the amount of carbon dioxide per hour produced during fasting with muscular relaxation and quietness with the amount produced after feeding when there was considerable extraneous muscular activity, as otherwise the effect due to the ingestion of food would be greatly magnified. Conversely, if during the fasting period the dog was unusually restless, and after feeding was quiet, the increase due to the ingestion of food would be too small.

Plan of investigation. The investigation began the end of May, 1911, and continued almost uninterruptedly until the middle of July, 1911. Since the apparatus was in use nearly every day for experiments with the two animals with atrophied pancreas, it was impracticable to conduct simultaneous experiments with a control animal. Furthermore, it was believed that the researches of Rubner, and more recently of Lusk, were practically identical in the results obtained with normal dogs, so that we could properly use these values for comparison with our results. Subsequently it was considered advisable to conduct control experiments with a dog of essentially the same body-weight as that of the dogs previously used and with the same apparatus, obtaining particularly the records of the muscular activity, since neither Rubner nor Lusk had controlled their experiments with such records. These control experiments were carried out in the fall of 1911 with a normal dog, "Clara."

After the completion of the experimental period with the normal dog, it was seen that the computation of the energy output of these dogs from the carbon-dioxide output might be open to the objection that while the normal dog after being fed with meat might be burning pure protein, the dogs which had been operated upon might be burning both protein and fat; under these conditions the calorific equivalent of the carbon dioxide would be very different, so that the results would not be comparable. It became necessary, therefore, to measure the oxygen consumption in order to determine the character of the catabolism of the dogs after operation. Accordingly, in the fall of 1912, modifications were made in the Benedict and Homans apparatus by means of which accurate determinations of the oxygen consumption could be obtained in addition to the determinations of the carbon-dioxide production. The accuracy of such measurements was frequently checked by burning alcohol inside the chamber and determining the respiratory quotient, the results obtained in a considerable number of half-hour periods showing a respiratory quotient for alcohol of 0.66 or 0.67, the theoretical value being 0.666. We therefore felt justified in using the apparatus for measuring the oxygen consumption, and this was done during the fall of 1912 in a series of experiments with "Flora," another dog in which all pancreatic juice had been excluded from the intestine.

General routine of experiments. In the fasting experiments, which were taken as a base line, the dogs were brought to the laboratory in the morning approximately 20 to 24 hours after the last meal, this meal usually consisting of 300 grams of meat and 300 cc. of milk. After the dogs had been weighed, they were placed inside the cage in the respiration chamber, the cover of the chamber was put on, and the ventilating current of air started. The experiment was then continued for six or more 4-hour periods, the carbon-dioxide production being measured for each period. Unless otherwise stated, the temperature of the air in the chamber was between 25° and 27°C., the experience of Benedict and Homans having shown that 25°C. is usually the optimum temperature for dogs. The feeding experiments were carried out according to the same routine except that just prior to the experiment, the dog was fed meat varying in amount from 500 to 1000 grams.

Calculation of the results. The total carbon-dioxide excretion was measured in all cases but, instead of presenting the data on the basis of the carbon-dioxide excretion per kilogram of body-weight, we have taken the average weight of the dogs as 7 kgms. and computed the results on the basis of a 7-kgm. dog. Since all four dogs used in this research were of nearly the same weight, i.e., about 7 kgms., this method of computation was even more satisfactory than if there had been a great variation in weight. Furthermore, the discussion of the results is wholly upon a *comparative* rather than an absolute basis.

Respiration experiments with the dog "Zep."

Experiments without food. Eight fasting experiments were made with the dog "Zep," the results of which are given in table 2. It was necessary to reject at least three periods¹⁹ on account of the gross muscular activity indicated by the kymograph records, and the results for these periods are not included in the average for the experiments. In all of the experiments, with the exception of the last two, the average carbon-dioxide production per 4 hours remained reasonably constant, ranging from 19 to 22.2 grams. The last two experiments, which are characterized by a continued

¹⁹ In many periods the dog was so restless as not to warrant a measurement of the carbon dioxide.

decrease in body-weight, showed a somewhat less carbon-dioxide production per hour. When computing the results on the basis of a 7-kgm. dog, the variations are from 18.1 grams to 21.8 grams, the average for the first seven experiments being 20.4 grams of carbon dioxide per hour. The low values found during the two experiments in July may possibly be accounted for by the excessive heat at that time and by the growing weakness of the animal. Since, however, the number of fasting experiments was approximately that of the feeding experiments, and they covered chrono-

TABLE 2.

Carbon-dioxide production in experiments without food with the dog "Zep."

EXPERIMENT NO.	DATE	BODY-WEIGHT WITH- OUT FOOD	CARBON-DIOXIDE PRODUCTION IN 4-HOUR PERIODS						AVERAGE CAR- BON-DIOXIDE PRODUCTION IN 4 HOURS	
			First period	Second period	Third period	Fourth period	Fifth period	Sixth period	At actual body- weight	On basis of 7 kilos body- weight
	1911	kilos	grams	grams	grams	grams	grams	grams	grams	grams
3	May 29		22.1						22.1	21.5*
4	31	7.19		21.6	21.4				21.5	20.9
8	June 7	7.27	21.1	19.2					20.2	19.4
13	13	7.12	23.5	22.0	21.1				22.2	21.8
18	20	6.96	23.3	16.8	16.9				19.0	19.1
24	27	6.92	24.7	19.9	20.2				21.6	21.8
30	July 5	6.66			16.1	17.9	17.0	17.6	17.2	18.1
	Average								20.5	20.4
37	July 13-14	6.44	12.2	15.3	15.4	15.8	13.5		14.4	15.7

* Calculated by means of weight assumed from May 31.

logically about the same time, we believe that the average results of all the fasting experiments may properly be compared with the average of the feeding experiments; hence the average fasting value of 20.4 grams for this dog is taken for a basal value.

One exception to this is made in the case of the fasting experiment of July 13-14. The low value then obtained, *i.e.*, 15.7 grams of carbon dioxide for 4 hours on the basis of a 7-kgm. dog has been used for comparison with a feeding experiment on the following day. For this reason the value for the experiment of

July 13-14 is not included in the average and the results are given separately in the table.

Feeding experiments. In the feeding experiments the animal was given at one meal the total amount of meat ingested just prior to entering the respiration chamber. The measurements of the carbon-dioxide production were made in periods of four hours each, and in some instances when excessive amounts of meat were

TABLE 3.

Carbon-dioxide production in experiments with food with the dog "Zep."
(Calculated to basis of 7 kilos of body-weight.)

EXPERIMENT NO.	DATE	BODY-WEIGHT WITHOUT FOOD	MEAT EATEN	CARBON-DIOXIDE PRODUCTION IN 4-HOUR PERIODS					
				First period	Second period	Third period	Fourth period	Fifth period	Sixth period
	1911	kilos	grams	grams	grams	grams	grams	grams	grams
5	June 1	7.19	1000			35.1	31.5	29.6	29.2
7	5-6	7.27	1000	32.5	32.5	36.1	28.4	28.0	24.2*
11	10	7.34	500		31.4	21.5	20.7	24.5	
14	14	6.97	500	28.4	23.4	24.2	25.5	19.0†	19.0†
16	17	7.02	500	27.8		20.2	20.2	18.4	
19	21	6.63	500	26.9		24.3	21.2	18.2	
22	24	6.93	500	23.5	32.9	21.7	22.8	21.3	
26	29	6.66	500	29.0	28.9	27.0	18.9	16.7	21.3
28	July 1	6.66	750	28.5	35.5	30.9	27.7	23.5	24.5
32	7	6.58	500				22.4	17.7	17.4
34	10	6.49	750						21.7‡
38	14-15	§	750	27.5		26.5	24.8	20.9	

* The production for the seventh period was 24.5 grams and for the eighth period 23.5 grams.

† Average production per 4 hours for a total of 8 hours. The results for two 4-hour periods have been combined because of a probably deficient absorber in the first of the two periods. The total carbon-dioxide measured was 37.8 grams.

‡ The production for the seventh period was 20.2 grams.

§ The weight used in calculating results to the 7-kilos basis was 6.44 kilos obtained on July 13.

given, the experiments were continued for thirty-two hours, or eight periods of four hours each. The data for the feeding experiments are given in table 3, which records the body-weight of the animal just prior to the ingestion of the food, the amount of meat eaten, and the carbon-dioxide production per 4 hours for the various periods. In tabulating these results, a relatively large number of periods were rejected owing to the fact that the kymograph records showed that the muscular activity was too great

to admit of any comparison with the results of the fasting period. It should be stated that this selection of periods by means of the kymograph records was made without reference to the results obtained in the measurement of the carbon-dioxide production and, indeed, the periods were rejected before a careful inspection of these data was made. Although the table is not meant for general comparison purposes, it can be seen from the results given that as a rule the larger the amount of meat fed, the larger the carbon-dioxide production.

TABLE 4.

Increase in the carbon-dioxide production following the feeding of meat in experiments with the dog "Zep."

(4-hour periods.)

EXPERIMENT NO.	DATE	MEAT EATEN	INCREASE* IN CARBON-DIOXIDE PRODUCTION							
			First period	Second period	Third period	Fourth period	Fifth period	Sixth period	Seventh period	Eighth period
	1911	grams	grams	grams	grams	grams	grams	grams	grams	grams
5	June 1	1000			14.7	11.1	9.2	8.8		
7	5-6	1000	12.1	12.1	15.7	8.0	7.6	3.8	3.1	3.3
11	10	500		11.0	1.6	.3	4.1			
14	14	500	8.0	3.0	3.8	5.1	-2.8			
16	17	500	7.4		-.2	-.2	-2.0			
19	21	500	6.5		3.9	.8	-2.2			
22	24	500	3.1	12.5	1.3	2.4	.9			
26	29	500	8.6	8.5	6.6	-1.5	-3.7	.7		
28	July 1	750	8.1	15.1	10.5	7.3	3.1	4.1		
38	14-15†	750	11.8		10.8	9.1	5.2			

* For fasting value for 4 hours, i.e., 20.4 grams (except July 14-15), see table 2.

† For July 14-15 the fasting value used is 15.7 grams obtained on July 13-14 and calculated to basis of 4 hours and 7 kilos of weight.

Increase in carbon-dioxide production following the feeding of meat. With the dog "Zep" the amount of meat fed varied from 500 to 1000 grams; the increments in the amount of carbon dioxide produced as a result of this feeding of meat have been computed and are given in table 4.

The average carbon dioxide per 4 hours in the fasting experiments being 20.4 grams, it will be seen from the results given in table 4 that in certain periods over 50 per cent more carbon dioxide was given off by the dog after eating meat than when fasting;

in fact, in the third period of the experiment on June 5-6, after the ingestion of 1000 grams of meat, the increase in the carbon-dioxide production was nearly 75 per cent, the increment being notable in all of the 4-hour periods in this experiment. The two experiments in which this large amount of meat was given showed that 1000 grams of meat were altogether too much to be ingested at one time, and thereafter the amount given was only 500 to 750 grams. With this smaller amount of meat, the increments were considerable during the first three or four 4-hour periods, but in no case when 500 grams of meat were ingested was the increase toward the end of the experiment of any size, usually reaching its limit at the fourth period. In the experiment of July 1, when 750 grams of meat were given, the increase in the carbon-dioxide production continued through all six periods, the rise and fall in the increased production being comparatively consistent. The same amount of meat was given on July 14-15, when the dog was obviously in a different condition physiologically; this, however, was not as successful as the first, inasmuch as the results of two periods were lost.

Total 24-hour increment in the carbon-dioxide production following the feeding of meat. While the increments for 4-hour periods are of interest in showing the time relations of the metabolism, the total effect of the meat feeding is best shown by noting the increment for the whole twenty-four hours. This has been computed for the experiments with the dog "Zep," and the results given in table 5. The increment shown is, in general, roughly proportional to the amount of meat ingested, although 750 grams may produce as great an increment as do 1000 grams of meat. The percentage of increment is of value for subsequent comparison with the results found in experiments with the normal dog "Clara."

Physical condition during observation. On May 31, 1911, about the time the experiments in the respiration apparatus were begun, the dog "Zep" weighed 7.19 kgms. When the feeding of the pancreas was stopped on April 15, the dog weighed 8.2 kgms. There was a gradual loss of weight from this time until the death of the animal and a lowering of the limit of assimilation for glucose, but diabetes did not develop. During the last two weeks of the experiments the dog appeared at times rather feeble and less playful. On July 18, after the completion of the respiration experiments, the dog weighed 6.19 kilograms. On August 18, the weight was 5.37 kgms. The observation was made at this time that she was thin but active and had

gained in strength during the previous month. In spite of the addition to the diet of fresh pancreas, which had been omitted during the respiration experiments, the dog slowly lost in weight and died on October 6, 1911. On the day preceding her death, she was out in the yard running about and was active and fairly strong, being able to jump into her cage which was elevated a foot or more from the floor. The last two weeks before her death, the stools were bulky, soft and fatty.

Results of autopsy. At the autopsy, no pancreatic tissue was found. The site of the corpus pancreatis was occupied by a small dense fibrous mass measuring about 2 cm. by 8 mm. by 2 mm., its outline merging into the surrounding connective tissues. The processus lienalis and processus uncinatus were entirely atrophied. A microscopic examination of the fibrous nodule showed scattered foci of epithelial cells in dense connective tissue but nothing that resembled pancreatic tissue.

TABLE 5.

Increase in the carbon-dioxide production in twenty-four hours following the feeding of meat in experiment with the dog "Zep."

EXPERIMENT NO.	DATE	MEAT EATEN	CARBON-DIOXIDE PRODUCTION IN 24 HOURS WITH-OUT FOOD	INCREASE OVER FASTING	
				Apparent actual Increase*	Proportion of Increase
	1911	grams	grams	grams	per cent
5	June 1	1000	122†	69‡	57
7	5-6	1000	122†	59	48
11	10	500	122†	24§	20
14	14	500	122†	20	16
16	17	500	122†	16**	13
19	21	500	122†	19**	16
22	24	500	122†	19	16
26	29	500	122†	24	20
28	July 1	750	122†	48	39
38	14-15	750	94††	53‡‡	56

* The amounts here given have been obtained by combining increases shown in table 4, excluding all minus quantities and also positive quantities of 1 grm or less.

† Obtained by multiplying by 6 the average results for 4 hours as brought to the 7-kilos basis (see table 2).

‡ From the results in the corresponding periods of June 5-6, it is assumed that the increase in the first and second periods of June 1 would total about 25 grams.

§ From the average of the increases for the first period on other days when 500 grams of meat were eaten, an increase of 7 grams is assumed for the first period of June 10. Because of increase above 1.0 gram in the fifth period, 0.3 grm increase in the fourth period has also been included in the total increase.

** From the average of the increases for the second period on other days when 500 grams of meat were eaten, an increase of 9 grams is assumed for the second period of June 17 and of June 21.

†† Obtained by multiplying by 6 the average result for 4 hours in the experiment of July 13-14 as calculated to the basis of 7 kilos of weight, i. e., 15.7 grams (see table 2).

‡‡ From the results in the first and third periods it is assumed that the increase for the second period would be 11 grams. The increase for the sixth period is assumed to be 5 grams.

Respiration experiments with the dog "Pat."

Experiments without food. Seven experiments without food were made with the dog "Pat," the results of which are given in table 6. The first six experiments were only twelve hours long and although the last experiment continued for twenty-four hours, it was necessary to reject the results of three periods on account of the muscular activity. In this table, also, the values are given for the average carbon-dioxide excretion during 4-hour periods, computed on the basis of 7 kgms. of body-weight. It will be seen that, on

TABLE 6.

Carbon-dioxide production in experiments without food with the dog "Pat."

EXPERIMENT NO.	DATE	BODY-WEIGHT	CARBON-DIOXIDE PRODUCTION IN 4-HOUR PERIODS						AVERAGE CARBON-DIOXIDE PRODUCTION IN 4 HOURS	
			First period	Second period	Third period	Fourth period	Fifth period	Sixth period	At actual body-weight	On basis of 7 kilos body-weight
	1911	kilos	grams	grams	grams	grams	grams	grams	grams	grams
6	June 2	7.27	21.7		21.8				21.8	21.0
9	8		25.9	23.6	20.8				23.4	23.1*
12	12	7.82		23.5	24.6				24.1	21.6
17	19	6.86	22.6	22.8	21.0				22.2	22.7
20	22	7.43	20.1	18.4	17.3				18.6	17.5
23	26	7.81	23.7	24.4					24.0	21.5
29	July 3	7.99				22.7	22.8	21.0	22.2	19.4
	Average								22.3	21.0

* Calculated by means of weight (7.09 kilos) assumed from June 9.

this basis, the average carbon-dioxide production in a 4-hour period for the seven experiments was 21 grams, the results ranging from 17.5 grams to 23.1 grams. On the other hand, the weight of the dog fluctuated, the lowest weight being 6.86 kgms. on June 19, and the highest 7.99 kgms. on July 3. With such sudden changes in the body-weight, it is to be questioned whether a computation can justly be made on this basis, although we are using this method of computation in practically all experiments. Since in the discussion of the results, we are dealing with averages rather than with individual experiments, it may be assumed with this dog

also that the average carbon-dioxide excretion in a 4-hour period, computed on the basis of 7 kgms. of body-weight, may be taken as the base line for comparison with the results of feeding experiments with the same dog, and in subsequent comparisons the value of 21 grams will be so used.

Feeding experiments. Nine feeding experiments were made with the dog "Pat," each of twenty-four hours' duration, in which amounts of meat varying from 500 to 750 grams were eaten by the dog immediately before he was placed in the chamber. The results, calculated on a 7-kgm. basis, are given in table 7.

TABLE 7.

Carbon-dioxide production in experiments with food with the dog "Pat."
(Calculated to basis of 7 kilos of body weight.)

EXPERIMENT NO.	DATE	BODY-WEIGHT WITHOUT FOOD	MEAT EATEN	CARBON-DIOXIDE PRODUCTION IN 4-HOUR PERIODS					
				First period	Second period	Third period	Fourth period	Fifth period	Sixth period
	1911	kilos	grams	grams	grams	grams	grams	grams	grams
10	June 9	7.09	500	29.4	29.2	26.1	27.8	21.9	20.4
15	16	7.22	500	26.8		26.7	29.9	23.7	23.0
21	22	7.43	500	25.2	29.3	26.1	25.1	19.7	18.4
27	30	7.85	750		26.2	30.1	26.9	24.2	23.5
33	8-9	7.91	500			21.9	20.2	17.5	17.3
36	12-13	*	750				23.5	21.1	17.0

* The weight used in calculating results to the 7-kilos basis is 7.85 kilos taken at 12 noon, July 11.

During certain days in July, the temperature of the air in the chamber rose considerably above 25°C., owing to the extreme heat at this time. Unfortunately with this dog a large number of periods, in fact, three entire experiments, had to be rejected owing to extraneous muscular activity, and it will be seen that in general all of the experiments after the first three were complicated by this excessive activity. It would appear from the results that the high temperatures incidental to the season were not well borne by the dog. While occasionally individual periods could be selected for computation of the results, on the whole the experimental evidence obtained after June 22 which can properly be used for discussion is very meagre. It should be emphasized here that during this whole time the dog was not so restless nor moved

about sufficiently to cause particular comment; the kymograph records, however, showed a continual disturbance and restlessness which in respiration experiments carried out with no records of the muscular activity would have been considered as entirely normal, and the results would have been used for drawing deductions. As previously stated, in no research thus far carried out in this laboratory have the kymograph records been of such value as in this study.

Total 24-hour increment in the carbon-dioxide production following the feeding of meat. The total increment in the carbon-dioxide production for twenty-four hours is of particular value for com-

TABLE 8.

Increase in the carbon-dioxide production in twenty-four hours following the feeding of meat in experiments with the dog "Pot."

EXPERIMENT NO.	DATE	MEAT EATEN	CARBON-DIOXIDE PRODUCTION IN 24 HOURS WITH-OUT FOOD	INCREASE OVER FASTING	
				Apparent actual increase*	Proportion of increase
	1911	grams	grams	grams	per cent
10	June 9	500	126†	29	23
15	16	500	126†	31	25
21	22	500	126†	22	17
27	30	750	126†	31	25

* The amounts here given have been obtained by the method used for "Zep" in table 5, i.e., by combining increases found for the 4-hour periods, excluding all minus quantities and also positive quantities of 1 gram or less.

† Obtained by multiplying by 6 the average result for 4 hours as brought to the 7-kilos basis; see table 6.

parison with the increment found with the normal dog; values for experiments after feeding 500 grams and 750 grams of meat were calculated on the same basis as those for "Zep" and are given in table 8. The noticeable feature of this table is the fact that the increase in the carbon-dioxide production after feeding 750 grams of meat is not appreciably greater than that found after feeding 500 grams.

Physical condition during observation. No sugar was found in the urine of this dog after the operation on February 13, 1911, but there was a progressive loss in weight after the extirpation of the pancreas on March 7, 1911, until April 4, 1911, when the weight had dropped as low as 7.15 kgms. From that time until the middle of August, 1911, there were only relatively

slight fluctuations, the range in weight being from 6.9 kgms. to 7.6 kgms. On March 25, 1911, the dog was found lying in the cage, being unable to stand on account of weakness. The feeding of pancreas was then begun and continued daily except on those days when the experiments were made in the respiration apparatus. After fresh pancreas was added to the diet, the dog gained slowly in strength; the stools continued soft and voluminous. On August 17, 1911, a record was made that the dog seemed fairly strong. During the following week the limit of tolerance for glucose was found to be about 15 grams. During the first week of September the dog vomited much of his food and grew quite weak. On September 10, 1911, he was found lying in the cage unable to rise. The animal was then killed with chloroform; after death, the dog weighed only 5.71 kgms.

Results of autopsy. An abscess was found in the lower part of the spleen measuring about 4.5 cm. by 3.5 cm. In the thick dense wall of this abscess was a wedge-shaped mass of fibrous tissue extending into the spleen for a distance of about 2 cm. There were no pancreatic remains at the site of the pancreas, but a microscopical examination showed a small amount of pancreatic tissue in the center of the fibrous nodule which extended from the abscess wall into the spleen. The pancreatic transplant, measuring about 3 mm. by 1 cm., consisted of scattered groups of acini separated and surrounded by connective tissue. No islands of Langerhans were found.²⁰

Control experiments with the normal dog "Clara."

It should be stated at the outset that if the experiments with the normal dog "Clara" had been made primarily for the purpose of studying the physiological effects of the ingestion of protein, a somewhat different plan would have been followed, but it seemed desirable to make these control experiments with so far as possible the same technique and routine as in the experiments with "Zep" and "Pat." The experiments should be considered, therefore, only as control experiments and not as a definite study of the metabolism of a normal dog as influenced by the ingestion of meat.

In the fall of 1911, a young, healthy, female dog, "Clara," was secured, weighing approximately 7 kgms. This dog proved to be a very satisfactory subject for experiments of this kind, as she became quickly accustomed to the technique and for the most part was very quiet, so that fewer periods were rejected on account of muscular activity than with any of the other dogs. The almost entire absence of muscular activity in the large apparatus was

²⁰ The histological details of the case are described by Pratt and Murphy: *Journ. of Exp. Med.*, xvii, p 252, 1913.

controlled by subsequent experiments in the small apparatus; in practically all experiments without food the dog had a minimum activity, the values found in both apparatus agreeing remarkably well.

Experiments without food. The results obtained in experiments without food and with the apparatus used for the experiments with "Zep" and "Pat" are given in table 9, and show that the average carbon-dioxide production for a 4-hour period on a 7-kgm. basis was 20.4 grams.

Feeding experiments. All of the experiments with food were made with the large chamber. Either 500 or 750 grams of meat were given the dog, since it was found impracticable to use as

TABLE 9.

Carbon-dioxide production in experiments without food with the dog "Clara."

EXPERIMENT NO.	DATE	BODY-WEIGHT	CARBON-DIOXIDE PRODUCTION IN 4-HOUR PERIODS				AVERAGE CARBON-DIOXIDE PRODUCTION IN 4 HOURS	
			First period	Second period	Third period	Fourth period	At actual body-weight	On basis of 7 kilos body-weight
	1911	kilos	grams	grams	grams	grams	grams	grams
41	Oct. 21	6.86	20.4				20.4	20.8
42	23	7.24		22.3	19.5	20.8	20.9	20.2
44	26	7.11	21.5	19.2			20.4	20.1
	Average						20.6	20.4

large an amount as 1000 grams, this amount being used only in the experiments with the dog "Zep." The results of the feeding experiments are given in table 10. While the data are not presented for the purpose of comparing the metabolism following the ingestion of varying amounts of meat, it will be seen that the results obtained with 500 grams of meat are approximately constant, and that on the one satisfactory day when 750 grams of meat were fed, namely, November 13-14, there was a much larger output of carbon dioxide than on the days when 500 grams of meat were fed.

Total 24-hour increment in the carbon-dioxide production following the feeding of meat. The total 24-hour increases found with the dog "Clara" are given in table 11, from which it is seen that the increase after feeding 750 grams of meat was greater than after

500 grams, the three experiments in which 500 grams were ingested agreeing very well.

TABLE 10.

Carbon-dioxide production in experiments with food with the dog "Clara."
(Calculated to basis of 7 kilos of body-weight.)

EXPERIMENT NO.	DATE	BODY-WEIGHT WITHOUT FOOD	MEAT EATEN	CARBON-DIOXIDE PRODUCTION IN 4 HOURS					
				First period	Second period	Third period	Fourth period	Fifth period	Sixth period
	1911	kilos	grams	grams	grams	grams	grams	grams	grams
45	Oct. 26-27	7.11	500	29.8	28.6	28.8	26.8	25.7	21.3
47	30-31	7.07	500	28.7	27.7	28.5	26.2	23.0	19.8
48	Nov. 2-3	7.06	500	32.1	28.5	27.5	27.3	19.6	20.0
54	13-14	6.95	750	31.0	37.5	33.5*	33.4*	24.9†	24.8†

* These results were calculated from the combined amounts for the third and fourth periods because of a deficient absorber in the third period.

† These amounts were calculated from the combined results for the fifth and sixth period because of a deficient absorber in the fifth period. The production for the seventh period was 26.2 grams when calculated to the basis of 4 hours and 7 kilos of weight.

TABLE 11.

Increase in the carbon-dioxide production in twenty-four hours following the feeding of meat in experiments with the dog "Clara."

EXPERIMENT NO.	DATE	MEAT EATEN	CARBON-DIOXIDE PRODUCTION IN 24 HOURS WITHOUT FOOD	INCREASE OVER FASTING	
				Apparent actual increase*	Proportion of increase
	1911	grams	grams	grams	per cent
45	Oct. 26-27	500	122†	38	31
47	30-31	500	122†	32	26
48	Nov. 2-3	500	122†	34	28
54	13-14	750	115†	71	62

* The amounts here given have been obtained by the usual method, i.e., by combining the increases for the 4-hour periods excluding all minus quantities and also positive quantities of 1 gram or less.

† Obtained by multiplying by 6 the average result for 4 hours as brought to the 7-kilos basis; see table 9.

‡ Obtained by multiplying by 6 the average result for 4 hours in the experiments of November 11 and November 13 with the small apparatus, i.e., 19.1 grams as brought to basis of 7 kilos of body-weight.

Respiration experiments with the dog "Flora."

The experiments with this dog were all carried out in the fall of 1912, immediately following the development of a method for determining the oxygen in the respiration apparatus, so that the amount of oxygen measured could be obtained as well as the carbon dioxide produced. It is unnecessary to give here the method of determining the oxygen consumption other than to say that it was based upon the fundamental principle carefully worked out for the large respiration chamber in this laboratory.²¹ The small apparatus with a capacity of 280 liters was used in all of the experiments with this dog, the respiration chamber being surrounded with a water bath which was kept by a Reichert thermostat at a temperature of 29° to 30°C.; under these conditions, the animal lay perfectly quiet for an hour or more at a time, so that sharply defined periods were easily obtained. The dog was unusually tractable, very quiet, and an ideal subject in every way. No long experiments were made, as the primary object of the study was the determination of the character of the catabolism.²²

Experiments without food. During the fall of 1912, a number of short-period experiments were made with this dog after it had fasted sixteen or more hours. The results of five of these experiments, each of which followed a fast of twenty-four hours, are given in table 12, showing an average carbon-dioxide production during 4 hours of 17.2 grams.

Feeding experiments. In the feeding experiments, instead of keeping the dog in the chamber continuously as was done with "Zep" and "Pat," the animal remained in the chamber only when she was being studied after the feeding. Satisfactory results were obtained on four different days with this dog, 750 grams of meat being fed in all instances, excepting on November 20, when only 700 grams were fed. The results for these experiments are given in table 13.

Increase in carbon-dioxide production following the feeding of meat. Assuming that the fasting base line for this dog on the basis

²¹ Benedict and Carpenter: Carnegie Institution of Washington, Publication No. 123, 1910.

²² We are indebted to Dr. Sergius Morgulis of the Nutrition Laboratory staff for valuable assistance in these experiments.

of 7 kgms. of body-weight was 17.2 grams of carbon dioxide per 4 hours, we can readily compute the increment due to the ingestion of meat. Thus, in table 13 we find three values for the first

TABLE 12.

Carbon-dioxide production in experiments without food with the dog "Flora."

DATE	BODY-WEIGHT WITHOUT FOOD	AVERAGE CARBON-DIOXIDE PRODUCED IN 4 HOURS CALCULATED ON A 7-KILOGRAM BASIS OF BODY-WEIGHT
1912	kilos	grams
Nov. 9	7.12	15.6
11	6.67	18.8
16	6.54	16.6
19	6.64	18.1
22	6.30	17.0
Average.....		17.2

TABLE 13.

Carbon-dioxide production in experiments with food (750 grams of meat) with the dog "Flora."

(Calculated to basis of 7 kilos of body-weight.)

DATE	BODY-WEIGHT WITHOUT FOOD	CARBON-DIOXIDE PRODUCTION IN 4-HOUR PERIODS					
		First period	Second period	Third period	Fourth period	Fifth period	Sixth period
1912	kilos	grams	grams	grams	grams	grams	grams
Oct. 25	7.6	26.5	26.2				
29	7.4	27.1	28.1				
Nov. 14	6.56	30.4					
20*	6.63				21.9		21.3
Average		28.0	27.2	27.2	21.9	21.9	21.3
Increment over fasting†		10.8	10.0	10.0	4.7	4.7	4.1
Total 24-hour increment				44.3			

* Only 700 grams of meat were given on this day.

† For fasting value for 4 hours, i.e., 17.2 grams, see table 12.

4-hour period following the feeding of meat, with an average of 28.0 grams, an increment over the fasting value of 10.8 grams. For the second 4-hour period, we have an average of 27.2 grams or

an increment over the fasting value of 10.0 grams. No values were obtained for the third 4-hour period but, according to the usual custom, we have assumed the increment to be the same as in the preceding period, *i.e.*, 10.0 grams. In the fourth period, the increment was 4.7 grams, the same increment being assumed for the fifth period in which no observations were made. In the sixth and last period the increment was 4.1 per cent. These data show, therefore, that the base line was not reached even at the end of the twenty-four hours, there still being an increased carbon-dioxide production of about 4 grams above the normal. Computing the total increment in twenty-four hours as was done for the other dogs, we find it to be 44.3 grams. Since with a fasting value of 17.2 grams, the total carbon-dioxide output for twenty-four hours of a dog without food would be 103.2 grams, the increment of 44.3 grams after eating meat would be equal to approximately 43 per cent.

Respiratory quotients obtained before and after the feeding of meat.

It is obvious that the respiratory quotient, if exactly determined, is of value in these experiments even when the muscular activity in some of the periods was too great to admit of the results being used for comparison. These quotients were determined for a number of days on which the animal was without food, and are as follows:

October 21.....	0.85	November 12.....	0.71
22.....	0.78	16.....	0.70
23.....	0.74	19.....	0.77
28.....	0.79	21.....	0.77
November 9.....	0.77	22.....	0.83
Average.....		0.77	

According to these fasting quotients, the dog was evidently not living wholly upon fat and inasmuch as the nitrogen excretion did not indicate an excessive disintegration of protein, we can only infer that the dog must have had a fairly liberal supply of glycogen which she could use when other food was not available. If the catabolism were of fat with a small proportion of protein, we should expect quotients averaging 0.75 or below, and the fact that the average is somewhat above this indicates a combustion of glycogen.

The respiratory quotients after feeding are of particular interest inasmuch as the study with this dog was made especially for determining the character of the catabolism following food. The quotients obtained under these conditions were as follows:

October 25.....	0.78	November 14.....	0.78
	0.71		0.79
29.....	0.80	20.....	0.79
			0.76

Aside from the second value for October 25, namely 0.71, it will be seen that all the quotients lie very close to 0.79 which is approximately the theoretical quotient for the combustion of protein; we may accordingly assume that for the greater part of the time during which these experiments were made, the dog was using protein. Even if the protein supply were deficient, the catabolism could not have been exclusively of fat, since this would tend to lower the quotient materially below 0.79, and the energy if not supplied by the combustion of protein must have been furnished to an appreciable degree (at least 30 per cent) by carbohydrates.²³

The series of experiments with the dog "Flora," therefore, establishes the fact that the character of the catabolism after feeding, as shown by the respiratory quotient, was essentially the same as that indicated by the carbon-dioxide production of the normal dog "Clara," i.e., the combustion was in large part of protein, the balance of the energy undoubtedly being derived in part from fat and in part from glycogen. If, however, the dogs operated on had had a small storage of glycogen, as might naturally have been expected from their emaciated condition, the combustion would have been in part of protein and in large part of fat, so that the calorific equivalent of the carbon dioxide produced would be different; consequently, the values obtained in the experiments with the dogs operated on would not be comparable with those secured with the normal dog. Since, however, the respiratory quotients obtained in the experiments with "Flora" show that the character of the catabolism was unchanged by the operation, it is reasonable to believe that for purposes of comparison, the carbon-dioxide production may properly be taken as an index of the total catabolism in all of the experiments with the four dogs.

²³ Magnus-Levy: *Physiologie des Stoffwechsels*, in *von Noorden's Handbuch der Pathologie des Stoffwechsels*, i, p. 207. 1904-5.

Physical condition during observation. The dog was sick for a week following the operation on March 29, 1912, and ate but little. There was no glycosuria. On April 11, 1912, it was noted in the records that the dog was very active. A stool passed that day contained a large number of muscle fibers. By May 20, 1912, the weight had fallen to 8 kgms., and on July 22, 1912, it was down to 6.66 kgms. The limit of tolerance for glucose had fallen from above 100 grams to less than 40 grams by June 26, 1912. On September 6, 1912, the weight reached the lowest point, i.e., 6.13 kgms. A week later the feeding of fresh pig's pancreas was begun. The dog gained rapidly in weight, and when the feeding of pancreas was discontinued on October 21, 1912, she weighed 8.35 kgms. During the week that the respiration experiments were made, no pancreas was fed. On January 19, 1913, her weight was 7.15 kgms. Previous to this date she had been well and active, but when the dog was being taken from the weighing room to the roof, her head was crushed by the elevator. There was profuse hemorrhage from the mouth and she died almost instantly.

Results of autopsy. An autopsy was performed thirty minutes after the dog's death. There was some subcutaneous fat, the mesentery also containing a fair amount of fat. The scar of the operation showed only a very little thickening. The peritoneal cavity was dry and the serosa pale and glistening. About 6 cm. below the pylorus there were some adhesions between the duodenum and the adjacent loops of intestine, and some thin fibrous bands united the duodenum to the liver and the gall bladder. No tissue that could be definitely recognized as the pancreatic gland was found. At the site of the processus lienalis, near some large blood vessels that ran transversely in the mesentery, were small semi-opaque white nodules 1 to 2 mm. in size resembling fat tissue, but of firmer consistency, which formed a thin arborescent structure situated between the layers of the mesentery.²⁴ When the mesentery which extended from the duodenum to the splenic region was held to the light, this structure was brought plainly to view, and was found to be about 4 cm. long, 0.5 to 3 cm. in width, and not over 1 mm. in thickness. Nothing that resembled a duct was seen. This tissue could be traced to the wall of the duodenum where a suture surrounded by a little dense fibrous tissue 2 to 3 mm. thick was found. There was no trace of the corpus pancreatis. Near the former junction of the corpus pancreatis and the processus uncinatus was another silk suture in a small nodule of fibrous tissue. The duodenum was here bound to the large intestine by adhesions. Careful dissection failed to reveal any remains of the processus uncinatus, and none were found on histological examination.

Nitrogen in the urine excreted by the dogs.

During the research an attempt was made to collect the urine excreted in 24-hour periods and determine the nitrogen. Great difficulty was experienced in securing this separation, so that we

²⁴ On histological examination these nodules were found to have the structure of islands of Langerhans.

have no exact knowledge regarding the amounts. Furthermore, as the feces were voluminous and soft, there was undoubtedly at times contamination from this source. The determinations of the nitrogen excretion, which were made only for "Zep," "Pat," and "Flora," have no value other than to give the general impression that the dogs were not destroying an excessive amount of body-protein, which was fully confirmed by the series of experiments with "Flora" on days when she was regularly catheterized.

That the dogs did not store protein in the body after the ingestion of 750 grams of meat is shown by the 4-day absorption experiment with "Flora," November 12-15, 1912, in which she was given this amount of meat daily. The nitrogen in the food was 83.31 grams. The excretion of nitrogen in the feces was 35.18 grams, and in the urine, 48.15 grams, or a total excretion of 83.33 grams. There was thus a loss from the body of 0.02 gram of protein.

No great storage of protein was shown even when 1000 grams of meat were given daily. In a 3-day absorption experiment, May 24-26, 1911, "Zep" excreted in the feces 37.24 grams of nitrogen and in the urine, 44.42 grams, or a total excretion of 81.66 grams. The nitrogen given in the food was 84.84 grams, so that in this length of time the dog retained but 3.18 grams of nitrogen.

Discussion of results.

Gross metabolism of dogs without food. The average carbon-dioxide excretion of the dogs used in this research, when without food and muscularly at rest, was as follows:

	Grams of carbon dioxide in 24 hours.
"Zep"	122
"Pat"	126
"Clara"	122
"Flora" ²⁵	103

²⁵ The determinations were made with the small apparatus. For purposes of comparison several experiments without food in which the small apparatus was used were also made with the normal dog "Clara," although the results are not included in this publication. The average 24-hour production of carbon dioxide, as obtained from the results of these selected half-hour periods, computed on the basis of 4 hours and 7 kgms. of body-weight, was 118 grams. A careful inspection of the kymograph records

Although these values were all computed to a uniform basis of 7 kgms. of body-weight, they do not materially differ from the actual measurements as the weights of the dogs were in all cases not far from 7 kgms. The exceptionally low value of 94 grams for "Zep" on July 14-15 was not included in making this average as we have reason to believe that so low a value was distinctly abnormal for this dog. These average values show that the total metabolism was not abnormal with either "Pat" or "Zep" as they agree remarkably well with that obtained for the normal dog "Clara."

From the determinations of the respiratory quotient, and other considerations which have been outlined in the discussion of these quotients, it can be seen that the carbon-dioxide production may be logically taken as an index of the total catabolism. Using the calorific value of carbon dioxide with a respiratory quotient of 0.79 as equal to 3.0 calories per gram, we may compute approximately the total heat output by multiplying by 3 the weight of carbon dioxide produced. That this method of computation may properly be used for purposes of comparison is shown by the fasting values obtained by Williams, Riche, and Lusk²⁵ in the direct heat measurements recently made on dogs, since we find that the total carbon-dioxide production when multiplied by 3 gives almost exactly the calories found. As a matter of fact, the dog used by the investigators cited had on at least three of the five days, a respiratory quotient considerably lower than those commonly found by us with our dog "Flora," but it is probable that this value of 3 calories per gram of carbon dioxide is not far from correct and hence it is justifiable to use it in this connection. Multiplying the weight of carbon dioxide by the calorific equivalent of carbon dioxide under these conditions, namely, 3 calories per gram, we then have for our dogs the following calorie values:

shows that there was essentially the same muscular activity in the small as in the large chamber, and the measurement of the metabolism was on an average essentially the same for the 24-hour period. On the other hand, the dog "Flora" showed through all experiments a much lower muscular activity than did the dog "Clara," which easily accounts for her lower metabolism when compared with the other dogs. In fact, the records for the dog "Flora" show strikingly a much greater degree of muscular rest than any of the other dogs experimented with.

²⁵ Williams, Riche, and Lusk: this *Journal*, xii, p. 358, 1912, table 1.

	PER 24 HOURS	PER KILOGRAM PER 24 HOURS
	<i>calories</i>	<i>calories</i>
"Zep"	366	52
"Pat".....	378	54
"Clara"	366	52
"Flora".....	309	44

The value obtained for "Flora" is considerably lower than that for "Zep," "Pat," or "Clara," but we should emphasize the fact in this connection that the temperatures at which the measurements were made with "Flora" were invariably 2° or 3°C. higher than those used with "Zep" or "Pat," and the computations are based on *selected half-hour periods*.

It is of interest here to compare the results obtained on two dogs in Rubner's laboratory. According to Rubner's computations, one dog weighing 6 kgms²⁷ had a calorie output, fasting, of 51 to 58 calories per kilogram in twenty-four hours. For another dog weighing 5 kgms., Rubner²⁸ computed the fasting metabolism as 56 calories per kilogram.

The conditions under which the measurements were made in the two investigations cited varied somewhat from those in our experiments. In three of the experiments reported by Williams, Riche, and Lusk, when the extraordinarily low average value of 38.3 calories per kilogram per twenty-four hours was found, the temperature of the environment was the same as that in our experiments, *i.e.*, 26° and 27°C., but the dog was asleep in the respiration chamber. In our experiments, however, none of the measurements were made exclusively during a period of sleep, and there was some minor muscular activity in all periods. Information regarding the muscular activity of Rubner's dogs is entirely lacking, but the statement is made that the temperatures at which the measurements were secured averaged always about 30° to 31°C.

While it is impossible to explain the differences in results, and they are undoubtedly caused by several factors, of which two are temperature difference and muscular activity, it is important in

²⁷ Rubner: *Die Gesetze des Energieverbrauchs bei der Ernährung*, Leipzig, 1902, pp. 42-46.

²⁸ Rubner: *loc. cit.*, pp. 318 and 323.

this connection only to note that the results obtained with our dogs come within what may be termed normal limits. The lower values found for "Flora" as compared with those obtained with "Zep," "Pat," and "Clara," are distinctly due to several facts, i.e., that "Flora" was a remarkably quiet dog, quieter than any dog heretofore used in this laboratory as a subject; and that practically all of the experiments were of short duration and of selected periods of minimum muscular activity. The results are also in conformity with the values obtained with the normal dog "Clara" when the small respiration chamber was used, which were lower than those obtained with the larger chamber when the experiments lasted several hours and often the whole day. The experimental evidence having established the fact that "Clara" was a normal dog, the values obtained with her are used in this discussion as a base line.

Comparison of the increments in the carbon-dioxide production following the feeding of meat to three dogs with pancreatic achylia and to a normal dog. The fact that all three dogs after operation showed a reaction to the ingestion of meat did not by any means throw definite light upon the question at issue, namely, whether or not the increase in the metabolism following the ingestion of meat is due to the mechanical movement of the food along the intestinal tract, and it is only by comparing the data with those obtained with a normal dog that such information can be gained.

Having shown that the calorie value is proportional to the carbon dioxide, instead of expressing the increments in the form of calories, we may compare simply the percentages of increment in the carbon-dioxide output after the ingestion of meat. This comparison is made in table 14. Following the feeding of 500 grams of meat, the average percentage increase in the carbon-dioxide production was with "Zep," 17 per cent; with "Pat," 22 per cent; and with the normal dog "Clara," 28 per cent. After giving 750 grams of meat, there was an average increase with "Zep" of 48 per cent; with "Pat," 25 per cent; with "Flora," 43 per cent; and with the normal dog "Clara," 62 per cent. "Zep" was the only dog who was given 1000 grams of meat, the percentage increase in the carbon-dioxide production following the feeding of this large amount being 53 per cent. No similar experiments were made with the normal dog "Clara."

When these results are compared, it is at once seen that the increase in the carbon-dioxide production was much larger with the normal dog "Clara" than with the three dogs operated upon. This was particularly true when 750 grams of meat were fed, although also apparent when only 500 grams of meat were given. In fact, with "Clara" the increase following the feeding of 750 grams of meat was 9 per cent greater than the increase with "Zep" when given as large an amount as 1000 grams.

TABLE 14.

Comparison of the 24-hour increments in the carbon-dioxide production after feeding meat to "Zep," "Pat," and "Flora," and the normal dog "Clara."

(On the basis of 7 kgms. of body-weight.)

	"ZEP"	"PAT"	"FLORA"	"CLARA" (normal)
Fasting value (grams)	122	126	103	122
Increase following feeding of 500 grams of meat (per cent).	20 16 13 16 16 20	17 25 23		31 26 28
Average	17.	22		28
Increase following feeding of 750 grams of meat (per cent)	39 56	25	43	62
Average	48	25	43	62
Increase following feeding of 1000 grams of meat (per cent).....	53			

If the increase in the metabolism following the ingestion of meat were due to nutrients absorbed out of the food and carried to the blood cells, we should expect a lower increment in the metabolism of the dogs operated upon than with the normal dog, since so large a proportion of the food was not absorbed. Among the possible objections that may be raised to these experiments, and we recognize that many objections can be raised to them if they are to be used in any other manner than was here intended, it may

be reasoned that dogs in so emaciated a condition might rapidly store the protein ingested instead of burning it. Under these conditions, Rubner has shown that the storage of protein does not exercise an influence upon the heat production. As a matter of fact, however, the nitrogen balance experiments that have been made with these dogs have shown invariably either a nitrogen equilibrium or very slight gains or losses, indicating that in general the dogs are in nitrogen equilibrium. Certainly no evidence was obtained to show that there was a large storage of protein during the ingestion of meat, so that the lower metabolism obtained with the abnormal dogs could not have been due to this factor.

On the other hand, if the increase following the ingestion of meat were due in large part to mechanical action, we should expect much greater increases in the carbon-dioxide output with the dogs operated upon than with the normal dogs. The stools voided by these dogs were enormous and, according to this latter theory, the mechanical work required to pass the mass of unabsorbed food through the intestinal tract would result in an increased metabolism, unless compensated by some other powerful factor. This is not shown, however, to be the case as the metabolism was considerably lower under these conditions than with the normal dogs.

It may be argued that with the dogs operated on, one of the largest glands in the body—the pancreas—had ceased to act, and if glandular work plays a large rôle in increasing the metabolism after the ingestion of food, this absence of the pancreatic function would lead to a somewhat decreased metabolism. Glandular work cannot be estimated by our method and hence the results should be considered with this in mind. It would be necessary, however, to ascribe to the work of the pancreas an excessive influence upon the metabolism if its absence were not only to compensate for the large increase in the intestinal activity and supposed incidental heat production, but actually to depress the metabolism below the normal level.

The results of these experiments show that there is no large energy transformation incidental to segmentation, peristalsis, glandular activity of stomach, liver and intestine, and the movement of the unabsorbed food through the intestinal tract. The attempt to explain the increased metabolism following the ingestion of food by the theory that the increase is a consequence of such movements is, therefore, not justifiable.

THE INFLUENCE OF AGE AND OF DIET ON THE RELATIVE PROPORTIONS OF SERUM PROTEINS IN RABBITS.

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In carrying out the following analyses, I employed the method devised by Robertson¹ for the determination of the serum proteins by the use of the refractometer.

A. Experiments on rabbits of different ages.

The rabbits used in these experiments were bred from six females (presumably Belgians), and a black "Flemish Giant" buck. As far as possible, the conditions were kept constant throughout the six months over which the experiments extended. The rabbits were fed on grain and alfalfa hay. Before bleeding, the animals were deprived of food for twenty-four hours and of water for two hours at least. They were bled by cutting the carotid of one side, care being taken not to sever the trachea and oesophagus. The blood was received into a beaker, and defibrinated by shaking with glass beads; then immediately centrifuged for one hour and forty-five minutes. To prevent evaporation as far as possible, all the Erlenmeyer flasks used were provided with rubber stoppers, and when filtering, the funnels were covered with moistened filter papers.

The results of the analyses are given in Table I. A comparison of the figures indicates the following:

1. The percentage of total proteins, and the percentage of total albumins show a slight gradual increase with age, from 21 to 140 days. The last three experiments, including the two adult females, show again a decrease in the percentages. It should be noted that

¹ T. Brailsford Robertson: this *Journal*, xiii, p. 325, 1912.

38 Influence of Age and Diet on Serum Proteins

TABLE I.

RABBIT NUMBER	AGE	WEIGHT*	"INSOL- UBLE" GLOBU- LINS	TOTAL GLOBU- LINS	TOTAL ALBU- MINS	TOTAL PRO- TEINS	PERCENTAGE RATIO TO TOTAL PROTEINS		
							"Insol- uble" globu- lins	Total globu- lins	Total albu- mins
	days	grams	per cent	per cent	per cent	per cent			
			±0.04	±0.15	±0.2	±0.2	±0.4	±2	±2
1	21	180(5)	0.21	1.1	4.4	5.5	3.8	20	80
2	24	183(4)	0.21	1.0	4.6	5.6	3.8	18	82
3	25	355(4)	0.21	0.7	4.6	5.3	2.6	13	87
4	30	433(4)	0.28	0.9	5.1	6.0	4.7	15	85
5	32	206(4)	0.24	0.7	5.0	5.7	4.2	13	87
6	39	807(2)	0.27	1.4	4.8	6.2	4.4	23	77
7	39	405(4)	0.28	0.7	5.1	5.8	4.8	12	88
8	54	692(3)	0.31	1.2	5.6	6.8	4.6	18	82
9	54	985(2)	0.31	1.2	5.0	6.2	5.0	19	81
10	57	1215(2)	0.24	1.5	4.8	6.3	3.8	24	76
11	57	1156	0.31	1.7	5.4	7.1	4.4	24	76
12	87	1656	0.35	1.1	6.0	7.1	4.9	16	84
13	87	1475	0.34	1.9	5.2	7.1	4.8	27	73
14	87	1339	0.34	2.2	5.2	7.4	4.6	29	71
15	87	1694	0.28	2.1	5.4	7.5	3.7	28	72
16	87	1890	0.39	0.9	5.8	6.7	5.8	13	87
17	97	2020	0.34	1.5	5.3	6.8	5.0	22	78
18	97	2024	0.31	1.6	5.3	6.9	4.5	23	77
19	98	1812	0.24	1.6	4.8	6.4	3.7	25	75
20	98	1747	0.34	1.8	5.6	7.4	4.6	24	76
21	102	1637	0.28	1.9	5.2	7.1	3.9	27	73
22	109	1836	0.24	1.3	5.2	6.5	3.7	20	80
23	115	2118	0.21	1.5	5.6	7.1	2.9	21	79
24	115	2333	0.21	1.5	6.1	7.6	2.7	20	80
25	125	2286	0.27	1.7	5.8	7.5	3.6	23	77
26	125	2323	0.27	1.5	6.0	7.5	3.6	20	80
27	136	2035	0.28	1.6	6.4	8.0	3.5	20	80
28	138	2465	0.27	1.3	6.6	7.9	3.4	17	83
29	138	2375	0.24	1.7	7.1	8.8	2.7	19	81
30	151	2065	0.31	2.1	5.4	7.5	4.1	28	72
31	151	2630	0.28	1.8	5.8	7.6	3.7	24	76
32	158	3030	0.28	1.6	5.3	6.9	4.1	23	77
†33	(1 Yr.?)	3130	0.24	1.6	5.0	6.6	3.6	24	76
†34	(1 Yr.?)	2495	0.28	1.2	5.2	6.4	4.3	19	81
†35	(1 Yr.?)	2950	0.42	3.1	4.2	7.3	5.7	43	57

* The numbers in the parentheses in the column of weights indicate the number of rabbits used in the experiment, the weight given being an average of the total weights.

† The rabbits used in these experiments were adult females, and were suckling young at the time; the young were used in experiments 1 and 2, above.

‡ This rabbit was also one of the adult females used for breeding. On autopsy, she was found to have an abscess of the uterus, the organ being enormously distended so as to nearly half fill the abdominal cavity and containing the necrotic remains of three embryos.

Reiss² has also found that in human beings the concentration of the total proteins in the serum of sucklings is lower than it is in the serum of adults.

2. The percentage of total globulins also shows a slight increase, but with greater individual variations.

3. The percentage of the "insoluble" globulins shows no constant variation corresponding with the age of the animals.

4. Considering the ratios which the various proteins bear to the total protein content, we note the following: *a.* There is no correspondence between the ages of the animals and the variations of the relative proportions of the three groups of proteins.

b. Taking the averages of the figures in the last three columns of Table I, we have for the series:

"Insoluble" globulins.....	4.0	$\left\{ \begin{array}{c} 5.8 \\ 2.7 \end{array} \right\}$	(± 0.4)
Total globulins.....	21.0	$\left\{ \begin{array}{c} 29.0 \\ 12.0 \end{array} \right\}$	(± 2.0)
Total albumins.....	79.0	$\left\{ \begin{array}{c} 88.0 \\ 71.0 \end{array} \right\}$	(± 2.0)

The first figure opposite each group represents the average percentages; the figures in brackets the highest and lowest observed in any individual; and the figures following the plus and minus signs, the experimental error.

The average figures here are intermediate between those which Robertson³ gives for "normal" rabbits, and for rabbits which had fasted five days, but the variation between the highest and lowest percentages is greater; this is probably due to the fact that I have analyzed the sera of a much larger number of animals, and hence have encountered more instances of extreme variation from the mean.

c. It is of interest to observe that the proportion of globulin to albumins in the serum of the animal (No. 35) with the uterine abscess was enormously greater than that observed in any of the normal animals, although the percentage of total proteins was normal.

² E. Reiss: *Jahrb. f. Kinderheilk.*, lxx, Heft 3, 1909.

³ T. Brailsford Robertson: *loc. cit.*

B. Experiments on the effect of diet.

Six rabbits were fed on milk alone for a period of two weeks, a control series being fed on a mixed diet of grain and alfalfa hay during the same time. The rabbits used were of medium size. They were bled, and the blood centrifuged and analyzed, as in the preceding experiments. The milk-fed rabbits were very emaciated; they were found to have lost in weight an average of about 200 grams (partly to be accounted for by the fact that the stomach and intestine were practically empty).

TABLE II.
Rabbits fed on milk.

RABBIT NUMBER	"INSOL- UBLE" GLOBULINS	TOTAL GLOBULINS	TOTAL ALBUMINS	TOTAL PROTEINS	PERCENTAGE RATIO TO TOTAL PROTEINS		
					"Insoluble" globulins	Total globulins	Total albu- mins
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>			
	± 0.04	± 0.15	± 0.2	± 0.2	± 0.4	± 2	± 2
1	0.24	2.0	5.2	7.2	3.3	28	72
2	0.35	2.1	4.8	6.9	3.6	30	70
3	0.20	1.4	5.2	6.6	3.0	21	79
4	0.20	1.4	5.2	6.6	3.0	21	79
5	0.34	1.6	4.8	6.4	5.3	25	75
6	0.34	1.5	6.0	7.5	4.5	20	80
Average	0.26	1.7	5.8	6.9	3.6	24	76

Control series.

1	0.21	1.7	5.0	6.7	3.1	24	75
2	0.24	1.6	5.0	6.6	3.6	24	76
3	0.14	1.3	4.6	5.9	2.4	22	78
4	0.14	0.8	5.4	6.2	2.3	13	87
5	0.24	0.8	5.0	5.8	4.1	14	86
6	0.27	1.2	5.2	6.4	4.2	19	81
Average	0.22	1.2	5.0	6.3	3.3	19	81

Comparison of the results from the two series (see Table II), shows the following:

1. The average percentages of the total proteins, as well as of each group, is higher in the case of the milk-fed rabbits. This is

probably to be accounted for by the fact that during the last few days the animals did not drink much of the milk, and were in a fasting condition.⁴

2. The milk-fed animals show an increase in the relative amount of the globulins, over the control series; but this difference is only slightly more than twice the experimental error of the determination.

CONCLUSIONS.

1. The percentage of total proteins in the blood serum of rabbits increases with age between the ages of 21 to 140 days. Fully adult animals have a slightly lower content of proteins in their blood serum than animals which are between 100 and 150 days old.

2. The relative proportion of "insoluble" globulins, "soluble" globulins, and albumins in the blood serum of rabbits varies somewhat in different individuals (fasted for 24 hours). There is, however, no correspondence between the ages of the animals and the variations in the relative proportions of these proteins.

3. Adult (medium-sized) rabbits fed upon a diet composed exclusively of milk for a period of two weeks, yield serum containing a slightly higher percentage of proteins than rabbits fed for a like period upon a normal mixed diet of grain and alfalfa; but the relative proportions of the above-mentioned proteins in the serum of the milk-fed animals did not differ in any significant degree from the proportions observed in the normal animals.

4. One animal which exhibited a pathological condition (uterine abscess) yielded serum which contained twice the relative proportion of globulins observed in normal animals, although the percentage of total proteins in the serum of this animal was normal.

⁴ Cf. T. Brailsford Robertson: *loc. cit.*

TOXIC BASES IN THE URINE OF PARATHYROIDECTOMIZED DOGS.

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In a recent paper¹ I demonstrated the presence of methyl guanidine in the urine of a parathyroidectomized dog, and called attention to the presence of some other bases. It is the purpose of this paper to report the occurrence of methyl guanidine in the urines of each of five other parathyroidectomized dogs, and to describe some of the other bases more fully. Experiments have also been made to determine in what manner mercuric chloride might best be used as a precipitant for their isolation.

Since the isolation of toxic bases from a urine involves a large number of manipulations and often large precipitates, quantitative results are hardly to be expected. The urines were therefore run in pairs, and the same technique so far as possible applied to each. I hoped in this way to be able to attribute any pronounced differences in the bases of two urines, similarly examined, to variations in metabolism. Moreover, substances that agree in a number of their properties when present in small quantities in each urine, could be combined.

The urines of the first two parathyroidectomized dogs were treated separately and similarly as follows: After each micturition the samples, collected by drainage from the cage, were acidified with HCl to precipitate the kynurenic acid. After standing in the cold until complete precipitation occurred, the precipitate was filtered off and the filtrate treated with tannin, barium hydroxide, sulphuric acid, and lead oxide according to Kutscher's method² for

¹ This *Journal*, xii, p. 313, 1912.

² *Zeitschr. f. physiol. Chem.*, xlviii, p. 1, 1906.

the removal of proteins. The urines thus purified were treated with mercuric chloride and sodium acetate according to the method of Engeland.³ The mercury salts of the bases thus obtained were taken up in dilute hydrochloric acid, decomposed with hydrogen sulphide, and the mercuric sulphide filtered off. The filtrate containing the hydrochlorides of the bases was evaporated to a syrup, and the organic substances extracted with methyl alcohol and the insoluble portion filtered off. After evaporation of the methyl alcohol, those substances easily soluble in ethyl alcohol were taken up in this solvent and treated with an alcoholic solution of platinum chloride. The precipitate that formed was filtered off, taken up in hot water and decomposed with hydrogen sulphide. The platinum sulphide was filtered off, and the filtrate concentrated and treated with gold chloride (Fraction A). Likewise the platinic filtrate after evaporation of the alcohol was decomposed with hydrogen sulphide and the platinum sulphide filtered off. The aqueous solution thus obtained was concentrated and treated with gold chloride (Fraction B). Those substances difficultly soluble in alcohol were taken up in water and treated with absolute alcohol until no more precipitate formed. The precipitate was filtered off, taken up in water and treated with picrolonic acid in aqueous solution (Fraction C). The filtrate after removal of the alcohol was likewise treated with picrolonic acid (Fraction D).

From fraction A of urine 1, 0.7 gram of yellow needles melting after two recrystallizations at 200°C. was obtained. They appeared similar to those mentioned in my preliminary report. From urine 2, 0.5 gram of the similar needles melting after two recrystallizations at from 200° to 205°C. was obtained. A number of crystals from each sample intimately ground together became soft at 200°C. and did not melt until 206°C. They were very insoluble in cold water and not readily soluble in hot water. After the removal of the gold each gave the diazo reaction with diazobenzene-sulphonic acid and sodium carbonate. A weighed portion of the combined crystals was taken up in water and the gold removed as the sulphide. This was converted into free gold and weighed. The filtrate containing the bases was treated with picrolonic acid.

³ *Zeitschr. f. physiol. Chem.*, lvii, p. 49, 1908.

The precipitate thus obtained after recrystallization melted at 266°C.⁴

The gold salt.

0.4301 gm. gave 0.2132 gm. Au.

0.6008 gm. gave 0.2997 gm. Au.

0.4453 gm. at 21°C. and 732.5 mm. gave 23 cc. N.

The picrolonate.

0.2000 gm. at 22.5°C. and 739 mm. gave 43.8 cc. N.

0.1609 gm. gave 0.2762 gm. CO₂ and 0.0576 gm. H₂O.

	Calculated for C ₈ H ₁₁ N ₂ ·2(AuCl):		Found:
		a	b
Au.....	49.9	49.67	49.88
N.....	5.32	5.68	

	Calculated for C ₈ H ₉ N ₂ ·2(C ₆ H ₅ N ₃ O ₂):		Found:
		a	b
N.....	24.14		24.13
C.....	46.92		46.81
H.....	3.91		4.01

This substance appears from the diazo reaction, the melting points, and analyses, to be β -imidazolyethylamine. I am surprised at its presence among the substances readily soluble in alcohol since the hydrochloride of this substance is described as being sparingly soluble in alcohol.⁵

There next occurred in each urine fraction, a crop of rhomboid plates. They were recrystallized twice, and melted slowly between 241° and 245°C. Portions of the crystals from each urine were intimately mixed. These melted at 243°C. After another recrystallization their form changed to needles but the melting point was not altered. From each urine 0.3 gram was obtained.

From urine 1, 0.2259 gm. substance gave 0.1019 gm. Au.

From urine 2, 0.1845 gm. substance gave 0.0831 gm. Au.

	Calculated for C ₈ H ₁₁ NOCl·AuCl ₂ :		Found:
		a	b
Au.....	44.52	45.16	45.04

⁴ Thermometer readings are not corrected. The nitrogen was collected over 40 per cent KOH; barometer readings are taken from an instrument with a glass scale, reading directly; direct readings are given not corrected except where indicated.

⁵ *Zeitschr. f. physiol. Chem.*, lxxv, p. 504, 1910.

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The melting point is that of choline aurichloride and the gold content indicates a substance of equal molecular weight.

After the solutions became quite concentrated, a large quantity of needles and some plates separated out. After three recrystallizations they began to soften in the melting tube at 262°C., became almost fluid at 286°C. and melted rising in the tube with bubble formation at 292°C. to 296°C. On analysis the substance was found to be free from carbon and proved to be the gold salt of ammonium chloride.

Fractions B of urines 1 and 2, the filtrates from the platinum precipitates, gave after concentrating and treatment with gold chloride, each, a precipitate of yellow needles. These after one recrystallization melted sharply at 198°C. Urine 1 gave 2.3 grams; urine 2 gave 2.9 grams. They were more soluble in alcohol and ether than in water. The gold was removed from a portion of the united needles, and weighed; the filtrate was treated with picrolonic acid. The picrolonate melted sharply at 275°C. with decomposition and bubble formation.

The gold salt.

0.1602 gm. gave 0.0763 gm. Au.

The picrolonate.

0.2023 gm. at 22.5°C. and 745 mm. gave 53.5 cc. N.

0.0950 gm. gave 0.1490 gm. CO₂ and 0.0369 gm. H₂O.

	Calculated for C ₂ H ₃ N ₃ (AuCl ₆):	Found:
Au	47.7	47.6
	Calculated for C ₂ H ₇ N ₃ (C ₁₀ H ₈ N ₄ O ₃):	Found:
N	29.14	29.31
C	42.7	42.78
H	4.45	4.35

This substance is evidently methyl guanidine as shown by the properties and analyses of these derivatives. After no more methyl guanidine aurochloride separated out, the gold was removed as the sulphide from the mother solution and the filtrates united and treated with picrolonic acid. A dark red, very insoluble precipitate formed. By burning in a crucible it left magnetic iron oxide. A voluminous precipitate was next obtained which melted after

two recrystallizations at 346°C. The yield was slightly over 1 gram.

0.1126 gm. substance at 23.5°C. and 738 mm. gave 22.2 cc. N.
 0.1129 gm. substance at 16.25°C. and 745 mm. gave 21.0 cc. N.
 0.1439 gm. substance gave 0.2327 gm. CO₂ and 0.042 gm. H₂O.

Found:

	a	b
N.....	21.54	21.21
C.....	44.1	44.14
H.....	3.27	3.49

On burning in a porcelain crucible the substance emitted a strong peach-blossom odor and left no ash. It needs further study.

Fraction C, the alcoholic precipitate of the substances difficultly soluble in alcohol, contained a large quantity of inorganic salts. However from this fraction of urine 2 a picrolonate was obtained. After several recrystallizations, it darkened at 340°C. and did not melt below 360°C. The yield was about 2 grams.

0.1912 gm. substance at 22.1°C. and 741 mm. gave 35.1 cc. N.
 0.1713 gm. substance at 23.5°C. and 740.5 mm. gave 31.2 cc. N.
 0.2347 gm. substance gave 0.0644 gm. H₂O and 0.3704 gm. CO₂.
 0.1822 gm. substance gave 0.0530 gm. H₂O and 0.2847 gm. CO₂.

Found:

	a	b
N.....	20.25	20.04
C.....	42.9	42.6
H.....	3.07	3.25

This substance has about the same solubility as the picrolonate obtained by treating ammonium chloride with picrolonic acid. The latter however melts at 278°-280°C.

Fraction D gave a small precipitate with picrolonic acid which upon an attempt at recrystallization did not precipitate until the solution had almost evaporated to dryness. It cannot be studied until more substance is at hand.

The urines obtained from dogs 3 and 4 were treated similarly to those of dogs 1 and 2, with the exception that the removal of the proteins was omitted. After the removal of kynurenic acid, the urines were evaporated to a syrup, taken up in methyl alcohol,

filtered and again evaporated to remove the excess of HCl , and then taken up in water. This solution was treated with mercuric chloride and sodium acetate according to Engeland's method to precipitate the bases. The urine fractions were similar to those of urines 1 and 2. Fractions A are again the platinum precipitates of substances soluble in alcohol. Fraction B of each urine is the filtrate from this platinum precipitate. Fraction C is the alcoholic precipitate of substances insoluble in alcohol. Fraction D, the filtrate from this precipitate.

Fraction A of urine 2 upon concentration and treatment with gold chloride gave 0.6 gram of rhomboid plates which after two recrystallizations melted at 248°C .

0.1901 gm. substance gave 0.085 gm. Au.

	Calculated for $\text{C}_2\text{H}_{11}\text{NO AuCl}_2$	Found:
Au.....	44.52	44.7

From urine 4 only a few needles melting at 238°C . were obtained. The quantity was too small for purification and analyses. As the solutions concentrated a large quantity of the aurochloride of ammonia was obtained.

Fractions B, the filtrates from the platinum precipitates, were found to reduce a test portion of gold chloride. After study they revealed the presence of ferrous iron. They were therefore made alkaline with Ag_2O . (Ag_2O was used instead of other alkalis because it could be removed so readily.) The precipitate obtained was decomposed with H_2S and gave upon removal of the silver sulphide, acidifying with HCl and concentrating, a large crop of beautiful translucent green rhomboid plates. These were very soluble in water and alcohol, and gave with potassium ferricyanide, the reaction for ferrous iron. They contained a large quantity of water of crystallization. They were dried by standing in a desiccator over H_2SO_4 for several days. In a vacuum tube under a slow stream of dry air and at 120°C . they lost after one-half hour a portion of their water of crystallization.

2.0298 gms. substance lost 0.3611 gm. H_2O at 120°C .

0.2729 gm. substance gave 0.1350 gm. AgCl .

0.3500 gm. substance gave 0.1728 gm. Fe_2O_3 .

	Calculated for FeCl_2	Found:
Fe.....	44.08	34.53
Cl.....	55.92	45.8

Evidently all of the water of crystallization was not lost by the drying. The substance appears to be ferrous chloride. The form in which the iron was excreted into the urine is not known, but it appears that it might, in part, have been part of a protein molecule since urines 1 and 2, from which the proteins were removed, contained much less iron.

The filtrates from the silver precipitates, after the removal of the silver, gave upon treatment with gold-chloride crops of beautiful needles. These after recrystallization melted sharply at 198°C. They were soluble in water and more readily soluble in alcohol and ether. Urine 3 gave 2.2 grams, urine 4 gave 1.7 grams. After recrystallizing from alcohol ether and water, a portion was freed from gold and converted into the picrolonate. This melted sharply, rising in the tube at 275°C. with decomposition instead of at about 270° as described by Achelis.

The gold salt.

0.1031 gms. gave 0.4930 gms. Au.

The picrolonate.

0.1024 gms. at 25°C. and 742 mm. gave 27.4 cc. N.

	Calculated for $C_2H_7N_3(AuCl_4)$:	Found:
Au	47.7	47.81

	Calculated for $C_2H_7N_3(C_{12}H_9N_3O_3)$:	Found:
N	29.14	29.29

Their solubilities and melting point, the analyses, and the urine fraction from which they were obtained, characterize them as the gold salts of methyl guanidine. These crystals were followed in urine 4 by a small quantity of cubes and short rectangular prisms of a brown color. The quantity was too small for purification. The mother solutions from both urine fractions were united and the gold removed with hydrogen sulphide. The filtrates thus obtained were treated with picrolonic acid. About 0.8 gram of a substance precipitated which after two recrystallizations melted at 260°C. The substance was quite insoluble in water.

0.1783 gm. substance at 22.0°C. and 748.5 mm. gave 44.2 cc. N.

	Calculated for $C_2H_7N_3(C_{12}H_9N_3O_3)$:	Found:
N	27.97	27.73

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This substance agrees in melting point and nitrogen content with symmetrical dimethyl guanidine.

Fraction C, the alcoholic precipitate of substances insoluble in alcohol, gave with picrolonic acid a precipitate which after several recrystallizations exploded at 348°C.

0.0752 gm. substance at 23°C. and 732 mm. gave 14.9 cc. N.

0.1619 gm. substance gave 0.2474 gm. CO₂ and 0.0490 gm. H₂O.

0.1070 gm. substance gave 0.1635 gm. CO₂ and 0.0364 gm. H₂O.

	Found:	
	a	b
N	21.56	
C.....	41.7	41.74
H.....	3.43	3.81

This substance requires further study. It is not very insoluble in water and may not be pure. Other derivatives are being prepared.

Urine 5. In order to determine if the choline present in the preceding urines might have arisen from phosphatides in the urine, an attempt was made to isolate such a substance. The urine was therefore extracted with an equal volume of ether. Upon evaporation of the ether only a trace of a lipid substance was found. This gave a precipitate with cadmium chloride, but was too small to examine further. The urine was then carefully neutralized, evaporated to a syrup and again extracted with ether. Upon evaporation of the ethereal extract no better result was obtained. Since the choline bases are known to give mercuric chloride salts which are less soluble in alkaline alcohol than in alkaline water, the syrupy residue of this urine was taken up in a small quantity of warm alcohol and the hot solution saturated with mercuric chloride and potassium acetate.

It was then treated with a hot saturated alcoholic solution of mercuric chloride and potassium acetate. While warm the mixture, in a wide mouthed bottle, was permitted to evaporate and then placed in the cold. When precipitation was complete, the precipitate was filtered off, taken up in dilute HCl, and decomposed with H₂S. The mercury sulphide was filtered off and the filtrate strongly acidified with HCl. After standing two days the

kynurenic acid had all precipitated and was then filtered off. The filtrate was evaporated to a syrup. The residue was now extracted with methyl alcohol, filtered, the alcohol evaporated, and the residue again extracted with methyl alcohol. After another repetition of the process no more inorganic salts were present in the extract, with the exception of ammonium chloride. The methyl alcohol was removed and the residue taken up in ethyl alcohol. The alcohol was evaporated off and the process repeated until only those substances easily soluble in alcohol were dissolved. The solution was then treated with alcoholic platinum chloride and the precipitate, which formed, filtered off (Fraction A). The alcohol was evaporated from the filtrate, the residue taken up in hot water and the platinum removed as the sulphide. The filtrate thus obtained was concentrated and treated with gold chloride (Fraction B). As in the previous urines the substances not dissolved by the extracting alcohol were taken up in water and precipitated with alcohol. This gave a precipitate (Fraction C) and a filtrate (Fraction D).

Fraction A, the platinum precipitate, was taken up in hot water and the substances readily soluble filtered off, as division 1. Substances left undissolved were again extracted with hot water, and the solution filtered from the insoluble portion (division 2). Those substances still undissolved were suspended in hot water and the three divisions decomposed with H_2S . The platinum sulphide was filtered from each. Division 1 was treated with gold chloride and gave a precipitate of rhomboid plates weighing about 2 grams. After two recrystallizations it melted at 238°C . The gold was removed as the sulphide, converted into free gold and weighed. The filtrate from the gold sulphide upon treatment with picronic acid gave a precipitate. This after one recrystallization melted cloudy at 178°C . and decomposed at about 230°C . The substance was dried in a vacuum. At 115°C . it lost water of crystallization.

Gold salt.

0.7392 gm. substance gave 0.3393 gm. Au.

	Calculated for $\text{C}_8\text{H}_{12}\text{N}_2 \cdot \text{AuCl}_4$	Found:
Au.....	46.4	45.9

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The pierolonate.

0.0979 gm. substance at 15°C. and 748.5 mm. gave 17.2 cc. N.
 0.2253 gm. substance at 21.5°C. and 740 mm. gave 40.8 cc. N.
 0.2107 gm. substance gave 0.1140 gm. H₂O and 0.3978 gm. CO₂.
 0.1630 gm. substance gave 0.0884 gm. H₂O and 0.3088 gm. CO₂.

	Calculated for C ₈ H ₁₁ N(C ₁₀ H ₈ N ₄ O ₂):	Found:	
		a	b
N.....	20.05	20.26	20.07
C.....	51.57	51.49	51.67
H.....	5.44	6.05	6.06

The substance is neurine as shown by the properties and analyses of the two derivatives.

The second platinic division was treated with pierolonic acid. It gave a precipitate weighing 2.1 grams which upon recrystallization was fractionated into two portions. The first and larger portion after recrystallizing several times melted between 284°C. and 286°C. The substance is quite insoluble in water.

0.1779 gm. substance at 22.5°C. and 740.8 mm. gave 41 cc. N.
 0.1596 gm. substance at 22.0°C. and 742.0 mm. gave 36.8 cc. N.
 0.1526 gm. substance gave 0.0609 gm. H₂O and 0.2583 gm. CO₂.
 0.1715 gm. substance gave 0.0648 gm. H₂O and 0.2895 gm. CO₂.

	Calculated for C ₈ H ₉ N ₅ O(C ₁₀ H ₈ N ₄ O ₂):	Found:	
		a	b
N.....	25.2	25.45	25.54
C.....	46.00	46.17	46.06
H.....	4.35	4.46	4.23

The second and smaller fraction after three recrystallizations softened and drew together in the melting tube at 240°C. and melted at 264°C.

0.0979 gm. substance at 18°C. and 736.5 mm. gave 22.5 cc. N.
 0.1038 gm. substance gave 0.0391 gm. H₂O and 0.1732 gm. CO₂.

	Calculated C ₈ H ₁₀ N ₄ 2(C ₁₀ H ₈ N ₄ O ₂):	Found:	
		a	b
N.....	25.74	25.85	
C.....	45.84	45.51	
H.....	3.97	4.21	

These substances freed from pierolonic acid did not give the diazo reaction and their structure is unknown. When more material is obtainable they will be studied further.

The third division was treated with gold chloride. A large precipitate of needles was obtained. These after recrystallization melted at 292°C . and appear to be the gold salt of ammonium chloride. After the solution had become quite concentrated a small quantity of flat yellow needles was obtained. They were not identified.

Fraction B, the alcoholic platinum filtrate from the platinum precipitate after the removal of the alcohol and platinum, gave a solution which reduced gold chloride with avidity. It reacted with potassium ferricyanide for ferrous iron. It was therefore made alkaline with silver oxide, the precipitate filtered off, and the silver removed from both precipitate and filtrate. The solution obtained from the precipitate upon concentration gave about 2 grams of ferrous chloride, as shown by its solubility in alcohol and water and the ferricyanide reaction. The silver filtrate after removal of the silver was slightly acidified with HCl , concentrated and treated with picrolonic acid. A precipitate was obtained in the form of fine needles which after several recrystallizations came to a constant melting point at 284°C . after previous sintering at 247°C . The weight was nearly 2 grams.

0.1993 gm. substance at 23°C . and 743.8 mm. (corr.) gave 50.1 cc. N°

0.1009 gm. substance at 23°C . and 730.0 mm. (corr.) gave 26 cc. N°

After another recrystallization the nitrogen content did not change.

0.1514 gm. substance at 18°C . and 754 mm. gave 37.8 cc. N .

0.0947 gm. substance gave 0.0453 gm. H_2O and 0.1592 gm. CO_2 .

0.1003 gm. substance gave 0.0473 gm. H_2O and 0.1670 gm. CO_2 .

Calculated for $\text{C}_{10}\text{H}_{11}\text{N}_4(\text{C}_{10}\text{H}_7\text{N}_3\text{O}_2)_2$:		Found:		
		a	b	c
N.....	28.48	28.36	28.54	28.63
C.....	45.65	45.85	45.41	
H.....	5.58	5.38	5.27	

It would seem that a substance containing both a guanidine and an amino group, such as the calculated substance, should form a salt with two molecules of picrolonic acid. However the acidity of the solution, and the careful addition of the picrolonic acid,

* These nitrogen determinations were made by Mr. Jiklin.

may account for the precipitation of the substance as a monopicrolonate. In order to determine its molecular weight, an attempt was made to extract and weigh the picrolonic acid from a weighed portion after acidifying with HCl. But the hydrochloride of the base is also soluble in ether and sufficient came over with the picrolonic acid to make the result valueless. The picrolonate is very insoluble in cold water, and not readily soluble in hot water. The nitrogen and hydrogen contents place it among the substituted guanidines. A small quantity was taken up in hot water and treated with a saturated solution of picrolonic acid. A precipitate formed during the cooling. This was collected, dried and used for a nitrogen determination.

0.1169 gm. substance at 22.5°C. and 742 mm. gave 27.2 cc. N.

	Calculated for $C_8H_{11}N_2(C_{10}H_8N_4O_3)$:	Found:
N.....	25.59	25.86

This substance is no doubt the dipicrolonate of the above substance. The analyses point to the mono- and dipicrolonate of either guanidine-butylamine or perhaps methylguanidine-propylamine. What the structural formula may be can be decided, definitely, only after further study.

After standing a few days the solution gave another precipitate in the form of small mounds, orange colored on the surface, light yellow inside. Some green-yellow microscopic crystals were also present. After six recrystallizations they melted at 276°C. The yield of pure substance was about 1 gram.

0.1065 gm. substance at 17.5°C. and 747 mm. gave 27.6 cc. N.

0.1454 gm. substance gave 0.0558 gm. H_2O and 0.2291 gm. CO_2 .

	Calculated for $C_8H_7N_3(C_{10}H_8N_4O_3)$:	Found:
N.....	29.14	29.52
C.....	42.7	42.98
H.....	4.25	4.30

This substance agrees in melting point and analyses with the picrolonate of methyl guanidine. The filtrates, remaining from the recrystallizing methyl guanidine solutions, were concentrated. From them, besides more methyl guanidine, a picrolonate was obtained in the form of yellow-green microscopic needles. These

were recrystallized, and melted at 272°C.–277°C. They were very insoluble in water. The yield was more than 0.5 gram.

0.1427 gm. substance gave 0.0672 gm. H₂O and 0.2332 gm. CO₂.

0.1337 gm. substance at 16.5°C. and 750.8 mm. gave 32.2 cc. N.

	Calculated for C ₂ H ₅ N ₃ (C ₁₀ H ₇ N ₄ O ₃):	Found:
N.....	27.97	27.68
C.....	44.42	44.57
H.....	4.84	5.26

The analyses show it to be dimethylguanidine picrolonate and the melting point distinguishes it as being asymmetrical dimethylguanidine which melts between 275°C. and 277°C. The original solution from which the above three substances came gave another precipitate of microscopic crystals. After three recrystallizations, it came down in the form of larger red crystals. The yield was about 0.4 gram. They melted at 272°C.

0.1702 gm. substance at 17°C. and 747.5 mm. gave 45.5 cc. N.

0.1107 gm. substance gave 0.0414 gm. H₂O and 0.1650 gm. CO₂.

	Calculated for CN ₂ H ₃ (C ₁₀ H ₇ N ₄ O ₃):	Found:
N.....	30.35	30.49
C.....	40.83	40.65
H.....	4.02	4.19

This substance is doubtless guanidine picrolonate, as melting point and analyses show. The mother solution next gave about 0.2 gram of a picrolonate that decomposed in the melting tube at 119°C. It gave the reaction for neutral sulphur. A portion of the substance was allowed to stand in the sunlight for a day, after which it turned dark green. It decomposed in the air bath at 80°C. It is remarkable that this substance could have escaped decomposition from the manipulation, and reach this fraction. Finally a voluminous precipitate was obtained in the mother solution. After recrystallizing a few times it exploded in the melting tube at 348°C.

0.1094 gm. substance at 19.25°C. and 736.5 mm. gave 21.5 cc. N.

0.1023 gm. substance gave 0.0324 gm. H₂O and 0.1707 gm. CO₂.

After one more recrystallization the contents altered but slightly.

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0.1100 gm. substance gave 0.0391 gm. H_2O and 0.1806 gm. CO_2 .

	Found.	
	a	b
N.....	21.83	
C.....	45.52	45.1
H.....	3.55	3.98

This substance is not very insoluble in water. I am not certain as to its purity. The presence of ammonium chloride is suspected since upon drying in the vacuum tube at 120°C . a small white sublimation took place forming a film on the roof of the tube. Ammonium chloride would hardly be expected to be present in this fraction. The solution always had an acid reaction, with the exception of the time of its short exposure to Ag_2O ; and ammonium chloride was previously removed quantitatively with platinum chloride. Its presence can however be explained by absorption of ammonia from the air.⁷

Fraction D, the alcoholic precipitate of substances insoluble in alcohol, after treatment with picrolonic acid gave a voluminous precipitate which after nine recrystallizations came down as long silky needles. They exploded at 355°C . and contained 21.38 per cent N, 44.14 per cent C and 3.5 per cent H. The substance which was very insoluble in water was not identified.

Histological changes.

The histological observations are mentioned here in so far as they appear to contribute to the interpretation of the presence of the bases found. The most striking histological changes occurred in the blood, liver, kidney and brain. The blood of the vena cava and heart of all animals showed extensive ante mortem coagulation. White clots in several cases were continuous from within the heart chambers down the vena cava to its iliac bifurcation. They nearly filled the lumen of the vessel. Upon section of the liver, the vessels showed fragmented erythrocytes, many normoblasts, erythroblasts with mitotic nuclei, and a small proportion of cry-

⁷ Seven or eight dogs were stored in the room in which this work was carried on. The atmosphere was generally ammoniacal and often very strongly so. The acid solutions during manipulation had great opportunity to absorb ammonia. Sulphuric acid was spread about for a time but there was not sufficient room for these safeguards and they had to be abandoned.

throcytes that stained brilliantly in eosin; the remaining red cells in large areas were blood shadows. Each section of the liver and lung showed a number of large mononuclear cells with eosinophile granules. There were also present a larger number of large flat cells staining very intensely in eosin. These showed no definite granulation. In places they were found to line the smaller veins like endothelial cells. In these places no endothelial cells could be observed. The cells of the hepatic cords showed advanced fatty degeneration of the protoplasm. The nuclei of large areas had disappeared entirely in places where the cell form was fairly well preserved. Such areas were surrounded by circular areas of cells in which the nuclei had become densely stained clumps of chromatin. In the livers of four of the dogs only a diffuse chromatolysis could be observed.

All kidneys showed marked congestion and hemorrhage in the cortex, some anaemic, and others, congested medullae. Some glomeruli had lost Bowman's capsule and were hemorrhagic, others were markedly congested. In some of the convoluted tubes the epithelium had degenerated.

The spleen contained a large quantity of pigment. Some of the cells showed chromatolysis.

The lung showed oedema, congestion and the blood changes mentioned.

The brain sections, which I prepared in Professor Barrett's laboratory, showed cells in the motor areas with partial loss of Nissl substance and typical tetany nuclei. Various degrees of chromatolysis were also observed in these nuclei.

The intestinal tract besides marked congestion showed in the duodenum and pyloric end of the stomach disintegrating epithelial cells. Their nuclei were converted into solid deeply staining clumps. These appeared like those in the process of extrusion from the normoblasts.

Symptoms.

After the operations the dogs lived from three to five days. The wounds showed beginning healing and no infection. The postoperative period can be divided into two stages. The first stage was free from symptoms. The first portion of the second stage showed mild symptoms. The dogs were uneasy and excit-

able, and at times appeared markedly depressed. Their pupils were sometimes unevenly dilated, and their limbs showed tremors especially after slight exertion. The last portion of the second stage was introduced by mild convulsions. The animal would lie on its side, its limbs extended and rigid. The breathing was rapid and also deep. His trunk muscles showed tremors. At times, he was oblivious to his surroundings and at other times wide awake, and appeared anxious. Such a convulsion was generally followed by a period of lassitude and fatigue, but the limbs were always more or less rigid and showed intermittently violent tremors. Some animals recovered and for a number of hours appeared quite normal. The latter part of this stage was marked by severe tetany and clonic convulsions in which the animal struggled as if to free himself. Salivation always occurred at this stage. The breathing became difficult, as from severe constriction of the air passages, and the inspirations and expirations produced high pitched and loud sounds that could easily be heard in a neighboring room. The salivation, and breathing which gradually assumed the Cheyne-Stokes type, generally was followed by death within a few hours. In some cases the bladder was distended and full of urine, in others it was constricted until the cavity was nearly obliterated.

Discussion.

The occurrence of toxic bases in large quantities in the urines of parathyroidectomized dogs, observed for the first time in my work, warrants a discussion, especially since the current views regarding the function of the parathyroids make no allowance for their presence. All the urines studied contained methylguanidine. Where this substance was found in smaller quantities other guanidine bases were present, so that the excreted guanidine nitrogen approached a constant in all animals. In addition to these bases others were observed although not uniformly distributed. β -Imidazolylethylamine was found in three urines out of six, choline in three out of five and neurine in large amounts in one urine. In urine 5 two unidentified bases were found in the fraction where β -imidazolylethylamine was previously observed.

In order to determine whether any of these substances could have been split from larger molecules during analysis, the following experiments were performed.

Fifty grams of Witte's peptone were taken up in 95 per cent alcohol and the solution treated with a hot saturated alcoholic solution of potassium acetate and mercuric chloride. Some mercuric oxide formed. The mixture was hydrolyzed on the water bath under a return condenser for one-half hour, then treated with a hot saturated alcoholic solution of mercuric chloride and potassium acetate, and allowed to stand in a wide mouthed bottle for three days. A large proportion of the alcohol evaporated off. It was then placed in the cold for one day. The precipitate was filtered off and treated like that obtained from urine 5. No bases except ammonium chloride could be isolated. In order to learn if Witte's peptone would yield any bases if the hydrolysis were prolonged, the experiment was repeated, the hydrolysis lasting eight hours instead of one-half hour. Otherwise the manipulations were like those of urine 5. Besides ammonium chloride and ferrous chloride two bases were isolated from the fraction corresponding to fraction A of the urines, and another substance was found in fractions B, C, and D. There is evidence of the presence of other bases.

The picrolonate of the first substance decomposed at 130° C.

0.0965 gm. substance gave 0.0351 gm. H₂O and 0.1721 gm. CO₂.

0.0893 gm. substance at 22.5°C. and 734 mm. gave 20.2 cc. N.

	Calculated for C ₁₁ H ₁₁ N ₂ (C ₁₂ H ₁₁ N ₂ O ₃):	Found:
N.....	24.34	24.76
C.....	48.53	48.64
H.....	4.04	4.07

These analyses agree well with those calculated for methyl imidazol but more data is necessary for a positive identification.

The picrolonate of the second substance softened at 192°C. and decomposed at 238°C. It was freed from picrolonic acid and converted into the gold salt; these were needles, quite insoluble in water. They melted at 232°C.

0.0261 gm. substance gave 0.0120 gm. Au.

	Calculated for C ₁₁ H ₁₁ N(AuCl ₄):	Found:
Au.....	46.4	45.98

The substance appears to be neurine but requires further study.

The third substance became more insoluble in water with each evaporation of the alcohol during the removal of the inorganic

salts and ammonia, so that it became sparingly soluble in water. It formed a yellow-green solution from which it crystallized in long slender yellow needles. It is soluble in hot chloroform, soluble in alcohol and ether. It gives no insoluble gold, platinum or cadmium derivatives. It gives the iso-nitrile reaction for a primary amine but no insoluble benzoyl derivative. When the aqueous solution is made alkaline with sodium hydroxide cherry red develops which is intensified by standing or more rapidly by heating. If this test solution is heated a small precipitate forms, also an oil that smells strongly of mustard oil. A solution of the substance can be heated with mercuric oxide without change, but upon rendering it alkaline and again heating the odor of mustard oil is produced and the mercury blackens. The substance is auto-oxidizable with change of the sulphur containing group. Boiling water or hot dilute acids split it into an oil, soluble in the usual organic solvents but not in water, and a substance soluble in water and the ordinary solvents from which it crystallizes in needles. The latter give a copper derivative which melts at 237°C . Sufficient substance is at hand for perhaps a proper identification and the analyses will be reported when the study is completed. The substance is of interest because of its probable genetic relation to the guanidines. The presence of a similar substance in the urines of parathyroidectomized rabbits suggested to me the search for guanidines in these urines.

The interesting investigations of Vaughan and his co-workers demonstrate that bacterial protein, egg white, and Witte's peptone after alkaline hydrolysis yield a toxic substance soluble in alcohol.⁸ These observers found that the substance was precipitable by platinum chloride though not in crystalline form. They could isolate no bases from it⁹ and regard it a peptone.¹⁰ Although the work of these and other investigators has not yet demonstrated that the above substances can be split from Witte's peptone I can find no other reason for their occurrence than that they must have been present in combination in the original peptone. However the uniformity in composition of various samples of Witte's peptone may perhaps be questioned.

⁸ *Zeitschr. f. Immunitätsforschung*, i, 1909.

⁹ *Journ. Amer. Med. Assoc.*, April 22, 1905; *American Medicine*, x, p. 145

¹⁰ *Trans. Amer. Assoc. Physicians*, xxvi, p. 193, 1911.

It is to be noted that the treatment given Witte's peptone in these experiments was much more vigorous than that which the urines received. The first experiment with its comparatively vigorous treatment yielded no bases as are found in these urines. In the second experiment the prolonged hydrolysis produced what are apparently neurine and methyl imidazol, and a substance which may be related to the guanidines genetically. These experiments show that prolonged alcoholic alkaline hydrolysis of a fairly large quantity of a protein produces minimal quantities of basic substances. It is therefore evident that the small quantity of protein in the urines after its comparatively mild exposure during the manipulations, could not materially alter the yields of the bases found. It is regretted that imidazol derivatives were not suspected in these urines and that the diazo reaction was neglected both before and after the removal of the proteins. The bases found may be considered to have been excreted into the urine as such nevertheless.

The histological picture of cellular disintegration may account for the excretion of these bases as products of passive protein disintegration. But something must account for the initiation of the changes. For this reason two feeding experiments were performed, none of the other dogs having been fed the day before the operation or at any time after the operation. Experiment I was performed with the dog that gave urine 1. This animal after having recovered from a violent convulsive attack and appearing quite well was fed 300 cc. of fresh milk. He drank about 200 cc. Within thirty minutes he was again in convulsions which increased in violence with exceeding rapidity and lasted two hours, proving fatal.

Experiment II was performed upon dog 5. For two days this dog had no symptoms. I then gave him some fresh sterile beef broth. He drank about 50 cc. and showed no symptoms for thirty-six hours, when they were very mild. The dog showed no stupor, but irritability. He was again fed 25 cc. of the same broth diluted, forty-eight hours after the first feeding. Within one-half hour he had symptoms of stupor with the legs in tetany and the respiration labored. The tetany was so general that the heart beat was transmitted to the abdomen in such a way that this could be seen to throb with each systole. All the muscles

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were in severe tetany within two hours after feeding. The dog was in a stupor and had lost all volition and appreciation of his surroundings. The eyes were bulged out, and the pupils dilated. Six hours after this feeding he had passed 300 cc. of urine, and two hours after this was wide awake and panting, his jaws snapped involuntarily and rigidity of his limbs gave way to tremors. He gradually recovered, could stagger about and drink small quantities of water. This he did quite often. I left him at about one o'clock a.m. in this condition. By morning he had passed 200 cc. more urine and appeared quite well. His breathing was rapid and he appeared much fatigued and excited. By noon the tetany again set in and the convulsions increased in violence until four o'clock p.m. when he died.

These experiments show that digested proteins taken into the body have very toxic effects after parathyroidectomy. These toxic effects are due to products of intestinal and perhaps also products of parenteral digestion. Such products of digestion are normally placed in some cell molecule or stored up in some form. In the case of these animals they are free and act as toxins. In other animals where no feeding occurred the symptoms increased in violence with short intermissions until death. The violence of the symptoms doubtless followed the rate of disintegration of the body protein. This disintegration had perhaps two sources, the preparation of units to supply cells for regeneration (these could no more be used than those received from the food) and the disintegration of the famished cells. The pathological condition would thus appear to be a failure upon the part of the cells to build up their protein. This part of the metabolism of the cell is regarded as a function of the nucleus. These indications together with the formation of free nuclein elements point to a nuclein atrophy. The histological findings moreover show an active nuclein degeneration. The extensive coagulation of the blood coming from organs rich in cells and nuclei, indicates the presence of free nucleic acid in the circulation, since nucleic acid coagulates blood plasma in acid solution. The acidity of the blood is indeed indicated by the absence of iron in the erythrocytes of the blood of this region as well as by the presence of a small proportion of erythrocytes that stain intensely in eosin.

The parathyroid secretion, therefore, appears to be concerned

ON THE ACTION OF TISSUES ON HEXOSES.

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(Received for publication, May 2, 1913.)

The process of intermediate carbohydrate metabolism has been analyzed by three different methods. The first consisted in feeding the normal or diabetic individual or animal with carbohydrates and their derivatives, or substances closely related to them. The second method consisted in perfusion of normal surviving organs with the same substances, and the third was based on the action on carbohydrates of tissue extracts, or of tissues in a state of autolysis, in the presence of antiseptics. Each of the three methods has its advantages and disadvantages. The disadvantages of the first are obvious, as it does not permit of getting hold of all the intermediate products and rarely permits of a quantitative analysis of the products. The shortcomings of the perfusion method are in a lesser degree the same as of the previous, and in addition, as was pointed out recently by Embden and his co-workers,¹ even in experiments lasting only two or three hours the danger of bacterial growth in the circulating blood is considerable.

Finally, the third method seemed to be free of the faults of the first two, and seemed to offer the best conditions for quantitative analysis of all products formed in the reaction. For this reason, and for the reason of the comparative simplicity of the experiment, a great number of investigations have been carried out by this method. However, the greatest part of the work on glycolysis by tissues and tissue extracts suffered from either imperfect technique or from lack of critical analysis of the results of experiments. In reality, it was demonstrated that under the usual conditions, namely, in the presence of antiseptics, no actual destruction of the carbohydrate molecule takes place. Whenever there was noted in such experiments a decline in the reducing power of the sugar solution this was caused by condensation of the monosaccharide into a polysaccharide, perhaps a disaccharide.

¹ *Biochem. Zeitschr.*, xlv, p. 1 et seq., 1912.

Up to the present date a successful dissociation of a hexose molecule outside of the body was attained only under one definite condition, namely, by the use of leucocytes in a 1 per cent Henderson phosphate solution and in absence of antiseptics. In this manner it was possible to convert hexoses into *d*-lactic acid.

This observation seemed of considerable significance since many French writers, particularly Lépine² and Mayer,³ were inclined to attribute to leucocytes the function of sugar decomposition in the living organism. The experiments presented here were undertaken with the object of testing whether or not the power of converting hexoses into lactic acid was limited to the leucocytes. It was planned to subject the sugars to the action of tissues in a 1 per cent Henderson phosphate solution under perfectly aseptic conditions. It proved to be a comparatively easy matter to secure kidney tissue in a perfectly aseptic condition, but the attempts to perform similar experiments with other organs offered great difficulties, hence it was concluded for the moment to limit the experiments to kidney tissue.

The results of these experiments were identical with those in the leucocytes experiments. Through the action of the kidneys, *d*-glucose, *d*-mannose and *d*-fructose were converted into *d*-lactic acid. There was perhaps a slight difference in the quantity of sugar attacked in the two sets of experiments, the more powerful action being noted in the leucocytes experiments. The lactic acid, as in previously reported experiments, was identified as the zinc salt.

Controls with kidney tissue without the addition of the hexoses showed complete absence of lactic acid.

The fact that also by the action of kidney tissue the three hexoses yield the same *d*-lactic acid carries sufficient evidence to the effect that the mechanism of lactic acid formation is identical in all tissues.

For the bacteriological examination we are indebted to Dr. J. Bronfenbrenner.

EXPERIMENTAL.

Tissues. Rabbits were killed by exsanguination and the kidneys removed aseptically. These were reduced to small pieces and transferred to the flasks containing the sugar solution.

² *Le diabète sucré*, Paris, 1909.

³ *Arch. internat. de physiol.*, ii, p. 131, 1904.

Solutions. The sugars were dissolved in the least quantity of water and sterilized. The 1 per cent Henderson phosphate solution was sterilized separately and then mixed with the sterile sugar solutions.

Methods of analysis. Sugar was estimated by reduction of Fehling's solution; the reduced copper was determined by the Volhard method.

Lactic acid. The solutions freed from protein were extracted with ether in a von der Heide extractor. The details of the process have been previously described. The lactic acid was determined as the zinc salt.

Bacteriological controls. Aerobic and anaerobic cultures were made of all mixtures prior to analysis by Dr. J. Bronfenbrenner and only those which proved free from all contamination were used.

I. Experiments showing disappearance of sugars in mixtures of sugar and kidneys.

d-Glucose.

	SOLUTION USED	NH ₄ CNS	NH ₄ CNS FEH CO.	SUGAR	LOSS	PER CENT LOSS
	cc.	cc.		per cent		
a. At beginning of experiment.....	2	31.90	15.95	5.72		
After thirty-six hours.....	2	29.60	14.90	5.34	0.38	6.64
After hydrolysis.....	1	14.80	14.80	5.30		
b. At beginning of experiment.....	1	16.20	16.20	5.80		
After thirty-six hours.....	1	15.20	15.20	5.44	0.36	6.21
After hydrolysis.....	1	15.40	15.40	5.52		
c. At beginning of experiment.....	2	27.00	13.50	4.84		
After thirty-six hours.....	2	24.60	12.30	4.41	0.43	8.93

d-Mannose.

d. At beginning of experiment.....	2	27.60	13.80	4.32		
After thirty-six hours.....	2	25.00	12.50	3.85	0.27	6.28
e. At beginning of experiment.....	2	29.00	14.50	4.54		
After thirty-six hours.....	2	26.20	13.10	4.20	0.34	7.48

d-Fructose.

f. At beginning of experiment.....	2	31.80	15.90	5.47		
After thirty-six hours.....	2	29.60	14.80	5.05	0.42	7.68

Two kidneys from a rabbit removed aseptically and immediately taken up in 1 per cent Henderson phosphate solution, were allowed to stand at 37° for thirty-six hours, under exactly the same conditions as those to which sugar had been added. There was no measurable reduction of the Fehling's solution either before or after incubation.

II. Experiments showing the formation of *d*-lactic acid.

Glucose.

200 cc. of the glucose mixture (c, Experiment I) were extracted with ether and the lactic acid converted into the zinc salt. Yield, 0.4350 gram zinc lactate.

0.1416 gram of the recrystallized and air dried salt lost 0.0180 gram H₂O on drying at 110° = 12.70 per cent H₂O.

Calculated..... = 12.88 per cent H₂O.

0.1416 gram dissolved in 2 cc. water, total weight 2.2712 grams, gave a rotation in a 1 dm. tube of $\alpha = -0.40^\circ$

$$[\alpha]_D^{20} = -6.4$$

Mannose.

100 cc. of the mannose mixture (e, Experiment I) gave 0.1860 gram zinc lactate.

0.0964 gram recrystallized and air dried zinc salt lost 0.0126 gram

H₂O on drying at 110°..... = 13.08 per cent H₂O.

Calculated..... = 12.88 per cent H₂O.

0.0964 gram dissolved in 2 cc. water, total weight 2.069 grams, gave a rotation in a 1 dm. tube of $\alpha = -0.30^\circ$

$$[\alpha]_D^{20} = -6.4$$

Fructose.

100 cc. of the fructose mixture (f, Experiment I) gave 0.2083 gram zinc lactate.

0.1328 gram zinc salt lost 0.0168 gram water on drying at 110° = 12.60 per cent H₂O.

Calculated..... = 12.88 per cent H₂O.

0.0872 gram dissolved in 1 cc. water, total weight 1.8090 grams, gave a rotation in a 0.5 dm. tube of $\alpha = -0.16^\circ$

$$[\alpha]_D^{20} = -6.6$$

ON CHONDROITIN SULPHURIC ACID.

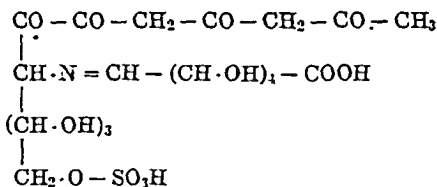
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(Received for publication, May 2, 1913.)

Chondroitin sulphuric acid has been the subject of repeated investigations, yet the information furnished by them added little to that advanced by the work of Schmiedeberg.¹ There is scarcely a new fact brought out by recent investigators which remained unchallenged and undisputed in the light of subsequent investigations.

According to the conception of Schmiedeberg the nucleus of chondroitin sulphuric acid is chondrosin. This, in its acetylated form (chondroitin) combines with sulphuric acid to yield chondroitin sulphuric acid.



The investigation here presented deals primarily with chondrosin. All writers are in accord in the view that chondrosin is composed of two substances in some way or other related to carbohydrates. According to Schmiedeberg the components are glucosamine and glucuronic acid. Orgler and Neuberg² contradicted Schmiedeberg on both points, claiming the components to be aminotetrahydroxycaproic acid and a hexose of undetermined configuration. S. Fränkel³ further modified the view of the two preceding writers in that he interpreted the nature of the nitrogenous body as that

¹ Schmiedeberg: *Arch. f. exp. Path. u. Pharm.*, xxviii, p. 358, 1891.

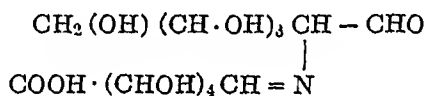
² Orgler and Neuberg: *Zeitschr. f. physiol. Chem.*, xxxvii, p. 407, 1903.

³ S. Fränkel: *Ann. d. Chem.*, cccli, p. 344, 1907.

of an aminoglucuronic acid, the other component supposedly having the structure of a very labile hexose with an undetermined configuration. Still later Kondo,⁴ working in Hofmeister's laboratory, reached the conclusion that one of the components was xylose. Finally reference has to be made to the observation of Mandel and Neuberg⁵ that chondroitin sulphuric acid gave a test with naphtoresorcin characteristic of glucuronic acid.

It must be added here that the only crystalline substances for which absolute purity was claimed by the writer were the salts of tetrahydroxyaminocaproic acid described by Orgler and Neuberg.

It is seen from this brief review that there exists an absolute lack of agreement on the nature of the components of chondrosin. No greater is the harmony of various writers on the mode of union of the two components. According to the view of Schmiedeberg, the carbonyl group of the glucuronic acid is attached to the amino group of the glucosamine, hence giving the following expression to the molecule:



Orgler and Neuberg criticized severely the conception of Schmiedeberg as inconsistent with the properties of the substance, one of the reasons being the observation of Schmiedeberg that the reducing power of a chondrosin solution is not diminished after oxidation with nitric acid; and also on the ground of the great resistance of chondrosin against the hydrolytic action of mineral acid. Orgler and Neuberg, however, do not advance a definite view on the mode of the union of the two components. Also S. Fränkel and Kondo furnish little information in that direction. The latter however argues against the presence of a free carboxyl group in the chondrosin molecule.

In course of the present investigations, which are as yet not completed, definite information was obtained of the nature of one of the components, and also of the condition of some of the characteristic groups of both components.

The difficulty of getting hold of the components is conditioned

⁴ Kondo: *Biochem. Zeitschr.*, xxvi, p. 116, 1910.

⁵ Mandel and Neuberg: *Biochem. Zeitschr.*, xiii, p. 148, 1903.

by the fact that chondrosin displays a great resistance towards usual hydrolytic agents, so that it was not possible to bring about hydrolysis of the molecule without simultaneously affecting the integrity of the components. This peculiarity of chondrosin was known to Schmiedeberg.

In course of the present investigation a method was found which brought about a cleavage of the chondrosin molecule permitting the isolation of at least one component. The method consisted in the use of sodium amalgam. The details of the method are described in the experimental part. The substance isolated was the usual glucuronic acid.

The substance was identified by the phenyl and parabromophenylhydrazine derivatives, by the fact that it yielded saccharic acid on oxidation with bromine, as well as by oxidation with nitric acid. The phenylhydrazine derivative had all the properties and the composition of the phenylhydrazid of the osazone. The substance was found to be identical with the one obtained under similar conditions from glucuron and first described by Thierfelder.

The *p*-bromophenylhydrazine derivative had the composition of the substance described by Guido Goldschmiedt and Ernest Zerner.⁶ Under the same conditions we were able to obtain the identical body from pure glucuron. In accord with Goldschmiedt and Zerner we were unable to obtain the substance described by Neuberg. However, the formation of a derivative with only one molecule of hydrazine to one molecule of glucuron is *a priori* not impossible. We made no special effort in obtaining it for the reason that Goldschmiedt and Zerner's substance formed very readily from both pure glucuron and from the hydrolytic products of chondrosin.

Regarding the mode of the union between the glucuronic acid and the second component it was established first that the glucuronic acid is not bound to the amino group of the second component for the reason that the presence of an unsubstituted amino group in the chondrosin was demonstrated by the nitrous acid process.

Further, it was also made obvious that the carbonyl group of the glucuronic acid does not take part in the linking of the two components. The reasons are the following. On oxidation of chondro-

⁶ Goldschmiedt and Zerner: *Ber. d. deutsch. chem. Gesellsch.*, xlv, p. 113, 1913.

sin with nitric acid a product is obtained which on distillation with hydrochloric acid gives rise to a minimal quantity of furfural, while the original substance yields a quantity required by a complex composed of one molecule of glucuronic acid and one of a carbohydrate of approximately equal molecular weight. The same oxidation product does not contain free saccharic acid, which could be identified as the acid potassium salt. However, the salt is readily obtained as soon as the oxidation product is hydrolyzed with alkali. Hence, the original oxidation product contains saccharic acid in conjugated form. This in its turn carries convincing evidence that the carbonyl group of the glucuronic acid is not the place of the union between the two components.

Whether or not the carboxyl group of the glucuronic acid is free or serves for linking the two components cannot be stated with certainty at present. Decision has to be postponed until the chemical nature of the second constituent is established. Chondrosin apparently contains only one free carboxyl group, and if the second component contains none then the conclusion will be obvious, that the carboxyl group of the glucuronic acid is present in the chondrosin molecule in a free state.

The fact that chondrosin contains both the carboxyl and the amino groups in a free state, while chondroitin sulphuric acid does not possess reducing properties, and does not react with nitrous acid to form nitrogen gas, indicates that both groups are combined in the more complex molecule with other radicals.

EXPERIMENTAL.

Preparation of chondroitin sulphuric acid barium salt.

Nasal septums of cattle were freed from bone and other extraneous material and ground through a meat chopper. Portions of 5 kgm. each were allowed to stand for two days with 10 liters of 2 per cent potassium hydrate solution. The extract was strained through a cloth and the residue again subjected to the same treatment with 5 liters of potassium hydrate solution and finally washed once with water. The united extracts were acidified with acetic acid and concentrated on the steam bath with an excess of barium carbonate to about half of their volume. The clear liquid was then poured off and the residue thrown on a folded filter and allowed

to drain off. This filtrate was united with the decanted liquid and the whole acidified with acetic acid and evaporated as before with barium carbonate to about 2 liters.

The separated protein and barium carbonate were removed by centrifugalization and the clear yellow liquid dropped into eight times its volume of glacial acetic acid and kept agitated by a turbine. The acid potassium salt thus obtained was filtered by suction, washed with glacial acetic acid and finally with alcohol and ether.

Two hundred grams of this product, which gave a slight biuret test, were dissolved in 10 liters of water and while the solution was kept stirred with a turbine, a solution of basic lead acetate was dropped in until complete precipitation had taken place. The lead salt, after having been washed several times by grinding in a mortar with water and filtering with suction, was suspended in 5 liters of water and with the addition of 100 grams of barium acetate and 50 cc. of acetic acid decomposed by long treatment with hydrogen sulphide with constant stirring. After standing for twelve hours the lead sulphide was filtered off and the slightly turbid solution of the barium salt precipitated by the addition of about one-third of its volume of 95 per cent alcohol. After filtering and washing with 50 per cent alcohol, then with 95 per cent, and finally with absolute alcohol and ether, the product after drying was a pure white powder, showing no trace of biuret.

This product is a mixture of the barium salts of chondroitin and chondroitin sulphuric acid. It showed no reduction of Fehling's solution and in the apparatus of Van Slyke no amino nitrogen.

0.5070 gram substance gave 7.75 cc. $\frac{N}{16}$ NH_3 .

0.4650 gram substance gave 7.40 cc. $\frac{N}{16}$ NH_3 .

0.4166 gram substance gave 0.1125 gram BaSO_4 .

	Calculated for $\text{C}_8\text{H}_{12}\text{NSO}_{11}$	Found:
N.....	2.01 per cent.	(1) 2.14 per cent.
		(2) 2.23 per cent.
S.....	4.60 per cent.	3.72 per cent.

Preparation of chondrosin.

Fifty grams of the barium salt of chondroitin sulphuric acid were dissolved in 150 cc. of equal parts of concentrated hydrochloric acid

and water, and heated for an hour on the water bath. Barium sulphate begins to separate at once, and after one hour the solution, which is only slightly colored, shows its maximum reduction of Fehling's solution, and all the nitrogen is present as amino nitrogen. The filtered solution was evaporated in vacuum to a very thick syrup and this was taken up in about 40 cc. of hot water and poured into 500 cc. of absolute alcohol. Partial precipitation of the chondrosin hydrochloric acid salt as a nearly colorless flocculent precipitate takes place. After standing over night, two volumes of absolute ether were added and the precipitate filtered with suction and thoroughly washed with absolute ether. For a final purification the product thus obtained is dissolved in about its own weight of water and precipitated and washed again as above described. It is a quite colorless powder, which when properly washed is not hygroscopic. The yield of the first product, dried over calcium chloride for two days in vacuum, was 27 grams.

0.1966 gram substance dried to constant weight at 100° gave 12.7 cc. amino N at 21°, 764 mm. N = 3.67 per cent.

0.3319 gram chondroitin sulphuric acid barium salt gave 5.8 cc. $\frac{N}{16}$ NH₃. N = 2.44 per cent.

0.5044 gram substance hydrolyzed for one hour with one part HCl and one part H₂O gave 18.3 cc. amino N at 16°, 760 mm. N = 2.11 per cent.

Cleavage of chondrosin with sodium amalgam.

Twelve grams of chondrosin hydrochloride in 100 cc. of water were allowed to stand with 100 grams of 2.5 per cent sodium amalgam. After about twenty minutes at ordinary temperature the solution takes on a bright yellow color and at the same time an evolution of ammonia begins. The solution is then neutralized with sulphuric acid and 100 grams of sodium amalgam are again added, the temperature always being kept at about 25°. After about one hour the solution is again acidified with sulphuric acid and allowed to stand over night, after the addition of a third 100 grams of amalgam. The solution is then separated from the mercury and filtered from the sodium sulphate with the addition of some animal charcoal.

Preparation of phenylhydrazine compound.

The solution obtained by the above treatment was diluted to about 200 cc. and after the addition of 15 grams of phenylhydrazine in 50 per cent acetic acid allowed to stand on the water bath. After about 20-30 minutes a dark tarry material has separated together with a small amount of solid material. At this point the solution is quickly filtered with suction on a hot funnel into a hot flask and the filtrate allowed to stand for two to three hours on the water bath. After this time the solution is filled with long yellow needles to which very little of the light-colored oil adheres. The crystals were filtered and washed with warm water and then with cold absolute alcohol until no more oil drops could be discerned under the microscope. When dried in vacuum the product melts with decomposition at about 115°. Attempts to recrystallize did not effect a purification and therefore the first product was used for the analysis.

0.1188 gram substance gave 0.2484 gram CO₂; 0.0634 gram H₂O.

0.1278 gram substance gave 19.2 cc. N, 17°, 758 mm.

	Calculated for C ₂₄ H ₂₅ N ₅ O ₇ · 1.5H ₂ O:	Found:
C.....	58.93 per cent.	58.80 per cent.
H.....	5.93 per cent.	6.12 per cent.
N.....	17.17 per cent.	17.33 per cent.

0.0599 gram substance in 5 cc. pyridine alcohol mixture rotated in a 0.5 dm. tube with D-light - 0.32°.

*Phenylhydrazine compound from glucuron.*⁷

One gram glucuron was warmed on the water bath for two hours with a little more than the required amount of normal sodium hydrate. The solution was neutralized with acetic acid, and 4 grams of phenylhydrazine in 50 per cent acetic acid and 4 grams of sodium acetate were added. After a short time crystallization of the phenylhydrazine compound in long yellow needles began and after three hours their amount had reached 1.6 grams. The material was purified by washing with cold alcohol and ether. It decomposed at about 115°.

⁷ Thierfelder: *Zeitschr. f. physiol. Chem.*, xi, p. 395, 1887.

0.0598 gram substance in 5 cc. pyridine alcohol mixture rotated in a 0.5 dm. tube with D-light — 0.322° .

By prolonged heating in vacuum at 100° the substance loses weight but before becoming constant decomposition sets in, while at lower temperatures no loss of weight was observed.

Parabromphenylhydrazine compound from chondrosin.

Twenty grams of chondrosin hydrochloride were treated in the usual way with sodium amalgam, and the resulting solution, after acidifying with acetic acid, heated on the water bath with 4 grams of parabromphenylhydrazine hydrochloride. After about one hour the solution was filtered from the separated tarry material and allowed to stand three hours longer on the water bath. The impure phenylhydrazine compound obtained was washed with alcohol until the impurities had been removed and then with ether. The substance may be recrystallized by dissolving in as little as possible of a mixture of one part of 50 per cent acetic acid and one part alcohol and then precipitating by the addition of two parts of hot water.

0.0568 gram of the substance in 5 cc. pyridine alcohol mixture rotated with D-light in a 0.5 dm. tube — 0.8° .

0.0614 gram twice recrystallized under the same conditions rotated — 0.75° .

0.1454 gram substance gave 12.5 cc. N, 22° , 762 mm.

0.1126 gram substance gave 0.0118 gram AgBr.

	Calculated:	Found:
Br.....	28.95 per cent.	27.00 per cent.
N.....	10.15 per cent.	9.72 per cent.

Parabromphenylhydrazine compound from glucuron.

One gram of glucuron in 100 cc. of water was heated on the water bath with 2.5 grams of parabromphenylhydrazine hydrochloric acid salt, which had been purified by twice recrystallizing from dilute hydrochloric acid and washing with ether, and 2.5 grams of sodium acetate. After about one hour 0.3 gram of a yellow crystalline substance had separated. The mother liquor filtered from the first crystallization gave upon further heating 0.2 gram more of the same substance. After recrystallization from 50 per cent acetic acid and alcohol it had the following composition.

0.1436 gram substance gave 13 cc. N at 22°, 758 mm.

0.1268 gram substance gave 0.0824 gram AgBr.

0.1338 gram substance gave 0.0124 gram Na_2SO_4 .

	Calculated for $\text{Br}_2\text{C}_{12}\text{H}_{17}\text{O}_4\text{N}_4\text{Na}$	($\text{C}_{12}\text{H}_{17}\text{N}_4\text{O}_7\text{Br}$):	Found:
Br.....	28.95 per cent.	20.97	27.65 per cent.
Na.....	4.17 per cent.		3.01 per cent.
N.....	10.15 per cent.	7.21	10.20 per cent.

0.0653 gram substance in 5 cc. pyridine alcohol mixture rotated in a 0.5 dm. tube with D-light — 0.90°.

Nitric acid oxidation of the products of hydrolysis of chondrosin.

Twenty-five grams of chondrosin hydrochloride were treated with sodium amalgam in exactly the same manner as described in the previous experiment. The solution, after having been freed from inorganic salts by precipitation with alcohol, was evaporated to a syrup. This syrup was quickly evaporated in a flat dish with nitric acid composed of one part of nitric acid, specific gravity of 1.42, and one part of water. The residue was then evaporated several times with water and finally taken up in 15 cc. of water and neutralized with potassium hydrate. Upon addition of glacial acetic acid the crystallization of the acid potassium saccharate began after a short time. After two days the yield amounted to 1.1 grams. For analysis it was recrystallized from water.

0.1253 gram substance gave 0.0427 gram K_2SO_4 .

	Calculated:	Found:
K.....	15.72 per cent.	15.32 per cent.

Nitric acid oxidation of chondrosin and subsequent hydrolysis.

Ten grams of chondrosin hydrochloride were evaporated in a flat dish on a water bath with 10 cc. of nitric acid and 10 cc. of water. The residue was dissolved in 10 cc. of water and 5 cc. of nitric acid and again evaporated to dryness. The final residue was then dissolved in 10 cc. of water and the solution divided into two parts of 7 and 3 cc. each and neutralized in the cold with potassium hydrate. The larger portion, after addition of 2 cc. of 50 per cent potassium hydrate, was allowed to stand for two hours on the water bath and then acidified with acetic acid. After several

hours the acid potassium saccharate began to separate. The yield amounted to 0.5 gram after two days. From the smaller portion, after addition of acetic acid, only a trace of the same substance separated after long standing.

0.1276 gram substance gave 0.0440 gram K_2SO_4 .

	Calculated:	Found:
K.....	15.72 per cent.	15.46 per cent.

Brom oxidation of the products of hydrolysis of chondrosin.

A solution of 25 grams of chondrosin hydrochloride was treated in the usual way with 25 per cent sodium amalgam. The solution was acidified with hydrochloric acid and allowed to stand for five days at ordinary temperature with an excess of bromine. It was then concentrated in vacuum to about 100 cc. and the principal amount of the salt separated by pouring the substance into hot absolute alcohol. The alcoholic solution was concentrated in vacuum to a syrup, taken up in water, and the halogen determined in an aliquot part. The requisite amount of lead acetate was then added to the remainder of the solution and the lead chloride and bromide removed by filtration. The excess of lead was then removed by hydrogen sulphide and the solution evaporated in vacuum to about 30 cc. It was then neutralized with potassium hydrate and after the addition of 10 cc. of glacial acetic acid allowed to stand for two days in the refrigerator. The separated crystals were filtered on suction and the product recrystallized from water. After drying it amounted to 1.6 grams.

0.1209 gram substance gave 0.0419 gram K_2SO_4 .

0.1210 gram substance gave 0.0466 gram H_2O ; 0.1242 gram CO_2 .

	Calculated for $C_8H_9O_5K$:	Found:
H.....	3.65 per cent.	4.28 per cent.
C.....	27.90 per cent. ³	27.98 per cent.
K.....	15.72 per cent.	15.55 per cent.

Furfurol from chondrosin after oxidation with nitric acid.

0.4219 gram of chondrosin (calculated from the nitrogen content) was evaporated to dryness with 5 cc. concentrated nitric acid and

³ Considering that one atom of carbon is contained in the ash as K_2CO_3 .

5 cc. of water. After repeated evaporation with water the solution of the residue was distilled in the usual way with hydrochloric acid of specific gravity 1.06, until no more furfurol was given off. Upon addition of 0.1 gram of phloroglucin 0.0076 gram of phloroglucoside was obtained, corresponding to 0.0218 gram of glucuronic acid, or about one-tenth of the amount present in chondrosin.

Desamido chondrosin.

Three grams of chondrosin hydrochloride in 50 cc. of water were treated with the calculated amount of silver nitrite (1.1 grams). After standing for several hours at ordinary temperature the reaction mixture was warmed on the water bath with occasional shaking. After the solution had been allowed to stand over night at ordinary temperature it was again warmed on the water bath for about two hours, after addition of 0.3 gram of silver nitrate and about 5 cc. of diluted hydrochloric acid. The excess of silver was then removed with a slight excess of hydrochloric acid and the solution evaporated in vacuum to a syrup which was taken up in very little water and poured into dry acetone. The gummy precipitate hardened quickly and was then ground with more dry acetone and washed with ether. The product was then a white amorphous powder resembling chondrosin in all its physical properties and its power to reduce Fehling's solution and gave the same amount of furfurol.

0.3710 gram substance dried at 100° in vacuum gave 0.0575 gram phloroglucoside corresponding to 0.1725 gram glucuronic acid.

ON THE SELF-DIGESTION OF THE THYMUS.

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The most abundant source of nucleic acid in the animal organism is the thymus gland. The occurrence of ferments in this gland which are capable of decomposing the nucleic acid contained therein, has been demonstrated by Kutscher¹ and by W. Jones.² One might naturally expect that by submitting the gland to self-digestion, all of the nucleic acid would be attacked and decomposed to a greater or less extent. In the case of autolytic enzymes occurring associated with proteins, an autolysis of the organ yields a mixture of products in which none of the original proteins can be detected. An aqueous extract of dog's liver is able to decompose thymus nucleic acid completely with the liberation of purine bases and phosphoric acid.³ In certain other organs intermediate products, such as nucleosides, result as the end products of digestion.⁴

Regardless of the length of time (up to two months) the thymus is submitted to self-digestion, a portion of the original nucleic acid remains unattacked. The question naturally arises as to whether this portion of the nucleic acid is identical with the original nucleic acid of the gland, or whether the residue is, in reality, altered by the ferments and constitutes some intermediate product in the decomposition. By boiling with dilute sulphuric acid, the portion of the molecule of thymus nucleic acid containing the purine bases is readily attacked, and a substance resembling nucleic acids but containing only pyrimidine bases remains intact.⁵ Furthermore, as is well known, in the hydrolysis of proteins with dilute mineral

¹ *Zeitschr. f. physiol. Chem.*, xxxiv, p. 114, 1901-02.

² *Ibid.*, xli, p. 101, 1904.

³ Amberg and Jones: *Ibid.*, lxxiii, p. 407, 1911.

⁴ W. Jones: this *Journal*, ix, p. 169, 1911; Levene and Medigreceanu: *Ibid.*, ix, p. 65, 1911.

⁵ This *Journal*, xii, p. 411, 1912.

acids or proteolytic enzymes, certain groups are removed much more readily than others and a residue remains which is very resistant to the further action of the hydrolyzing agent.⁶

A mixture of thymus and water was allowed to digest and was filtered. The filtrate gave no precipitate of purine bases on treatment with an ammoniacal solution of silver nitrate. On the addition of acetic acid, a copious white precipitate of nucleoprotein was produced, and after filtration the purine bases could be readily detected in the filtrate by means of silver nitrate and ammonia. This explains an observation that acetic acid, added to the products of the self-digestion of the thymus, removes or decomposes some substance which interferes with the precipitation of the purine bodies. The nucleoprotein, referred to above, is evidently responsible for this, as it is well known that proteins, nucleic acids, and other substances prevent the precipitation of purine bases by means of silver nitrate and ammonia.

From the nucleoprotein thus obtained, a nucleic acid can readily be prepared. Furthermore, the residue of undissolved glandular material remaining after digestion, yields another specimen of nucleic acid. The fact that these two nucleic acids do not consist of products intermediate in the decomposition of the original material but are identical with the same, is proven by a comparison of their properties and hydrolytic products with those of the thymus nucleic acid prepared from the fresh gland.

Hence, it appears that the ferments of the thymus cannot digest all of the nucleic acid of the gland, but leave a portion completely unaltered. Whether this is due to the fact that the initial ferment which decomposes the nucleic acid is not a true catalytic agent,⁷ to a destruction of the ferment, or to an inhibitory effect of the products of the action cannot be decided at present.

⁶ Hammarsten-Mandel: *Textbook of Physiological Chemistry* (sixth edition), p. 128.

⁷ The fact that certain ferments are not true catalytic agents, but that a given amount of ferment can decompose only a definite quantity of substrate and no more, has been shown in other connections: W. Jones: this *Journal*, xii, p. 34, 1912; Howell: *Amer. Journ. of Physiol.*, xxvi, p. 453, 1910; Bunzel: U. S. Dept. of Agric., Bureau of Plant Industry, Bulletin No. 238.

EXPERIMENTAL.

Three specimens of nucleic acid were obtained and their properties compared as described below. The phosphorus was determined in the usual manner by fusion with caustic soda and potassium nitrate. The ammonium magnesium phosphate was weighed directly as described by W. Jones.⁸ The rotation of a 1 per cent solution of each was determined in a 2 dm. tube, using Welsbach light. Since it was found that the amounts of purine bases yielded on hydrolysis depended somewhat on the conditions of hydrolysis, a specimen of each was treated in exactly the same manner. Three grams were boiled with 100 cc. of 5 per cent sulphuric acid for two hours under a return condenser. While still hot, the solution was nearly neutralized with caustic soda and made alkaline with ammonia. The guanine and adenine were precipitated as silver compounds, decomposed with hydrochloric acid, and estimated in the usual manner.

Specimen A. This was obtained from the fresh gland by Neumann's method.⁹ One kilo of gland yielded 33 grams.

0.600 gram gave 0.337 gram $\text{NH}_4\text{MgPO}_4 \cdot 6\text{H}_2\text{O}$. A 1 per cent solution rotated 1.25° to the right.

Specimen B. One kilo of finely ground gland, 3 liters of water, and enough chloroform to prevent putrefaction were digested at 38° for three weeks. The material was filtered, and the filtrate treated with acetic acid. The precipitate of nucleoprotein was filtered, and dried with alcohol and ether. The nucleic acid was prepared from this nucleoprotein by heating with caustic soda in the usual manner. Four to five grams were obtained.

0.500 gram gave 0.288 gram $\text{NH}_4\text{MgPO}_4 \cdot 6\text{H}_2\text{O}$ and with another specimen 0.400 gram gave 0.235 gram $\text{NH}_4\text{MgPO}_4 \cdot 6\text{H}_2\text{O}$.

A 1 per cent solution rotated in 2 dm. tube 1.20° to the right.

4.3 grams were heated with 25 cc. of 25 per cent sulphuric acid in an autoclave at 130° – 140° for two hours. The pyrimidine bases were isolated by the usual method.¹⁰ 0.225 gram thymine and 0.240 gram cytosine picrate were obtained.

⁸ This *Journal*, ix, p. 177, 1911.

⁹ *Arch. f. Anal. (u. Physiol.)*, 1899, Supplement, p. 552.

¹⁰ W. Jones: *Zeitschr. f. physiol. Chem.*, xxix, p. 461, 1900.

Specimen C. 240 grams of ground and trimmed gland, 720 cc. of water and enough chloroform to prevent putrefaction were digested at 38° for two months. The mixture was filtered and the residue on the filter treated according to Neumann's method for preparing thymus nucleic acid. 2.3 grams were obtained.

0.500 gram gave 0.308 gram $\text{NH}_4\text{MgPO}_4 \cdot 6\text{H}_2\text{O}$. A 1 per cent solution rotated in a 2 dm. tube 1.03° to the right.

Considering the fact that the materials were air-dried and contained probably different amounts of moisture, and further that no special attempt was made to purify the products, the following table indicates clearly the identity of the three specimens.

SPECIMEN	PHOSPHORUS PER CENT	GUANINE FROM 3 GRAMS	ADENINE PICRATE FROM 3 GRAMS	SPECIFIC ROTATION
A	7.13	0.158	0.532	+62.5°
B	7.38	0.160	0.520	+60.0°
C	7.82	0.130	0.416	+51.5°

It is, therefore, to be concluded that in the self-digestion of the thymus gland, the ferments of the gland are not capable of decomposing all of the nucleic acid within any reasonable length of time, and, moreover, the undecomposed portion of the nucleic acid appears to be identical with that prepared from the fresh gland.

ON THE PREPARATION OF TYROSINE.

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Owing to its great insolubility and ease of crystallization, tyrosine is one of the easiest products to isolate from a mixture of amino-acids. Its preparation from silk, however, according to the usual method is attended with considerable expenditure of time and labor and the use of large quantities of chemicals. The silk is hydrolyzed by boiling for eighteen hours with 25 per cent sulphuric acid, the mixture greatly diluted with water, and the sulphuric acid removed quantitatively with barium hydroxide. The precipitate of barium sulphate is then washed with several liters of boiling water, and the filtrate and washings (amounting to 15 liters or more) evaporated to crystallization. The product is generally highly colored, and needs to be recrystallized several times with the use of animal charcoal. A much less laborious method has been proposed by Abderhalden,¹ in which the hydrolysis is accomplished by fuming hydrochloric acid. The yield, however, is not as good as by the older procedure. Furthermore, raw silk is expensive as a starting material, and not always available. Considering the importance of tyrosine, especially its use in the preparation of certain synthetic polypeptides, it seemed desirable to find a method which would involve but little expenditure of time or material in its execution.

In the study of certain problems involving the self-digestion of a very concentrated pancreatic extract, it was observed that the fluid on cooling deposited beautiful white crystals of tyrosine. Since the tyrosine is very readily and quickly liberated from casein through the action of trypsin,² the use of this protein in connection with the pancreatic extract suggested itself at once. The concentrated pancreatic extract, whose preparation is described below, digests casein very readily both in the natural medium of acidity of the extract

¹ *Zeitschr. f. physiol. Chem.*, xlviii, p. 523, 1906; lxxvii, p. 75, 1912.

² Abderhalden and Voegtlin: *Zeitschr. f. physiol. Chem.*, liii, p. 315, 1907.

and in the mixture when made alkaline with ammonia. From such a mixture of casein and pancreatic extract, when digested at 38°, the tyrosine crystallizes, and can be readily obtained by filtration.

Finely ground and trimmed pig's pancreas is mixed with an equal weight of water and enough chloroform to prevent putrefaction, and allowed to stand at room temperature for two days. This is necessary in order that the chloroform may penetrate the gland; otherwise, putrefaction would result in such a concentrated mixture. The mixture is now placed in a thermostat and digested for twenty-four hours at 38°, cooled and filtered. The filtration proceeds quite slowly, but can be continued over night if necessary. 100-150 grams of casein³ are added to each liter of the clear yellow filtrate, the mixture rendered slightly alkaline with ammonia, and digested for any convenient period from three to seven days at 38°. On removal from the thermostat, the fluid is allowed to stand over night. It is then filtered, and the precipitate well washed with cold water. The tyrosine is separated from any undigested casein or insoluble impurities by extraction of the precipitate with boiling water. Three extractions are sufficient, using 1000, 500, and 250 cc. portions of water successively. The combined extractions are evaporated to a small volume (about 250 cc.) and allowed to cool, whereupon the tyrosine separates in crusts or macroscopic crystals. For further purification the product can be recrystallized from hot water in the usual manner. One liter of the pancreatic extract and 100 grams of casein yield about 5 grams of tyrosine, while 1 liter of the extract alone yields about 1.2 grams. Analyses of three typical preparations are cited below. The specimens were not recrystallized.

- I. 0.6783 gram required 37.52 cc. 0.1N hydrochloric acid (Kjeldahl).
- II. 0.7600 gram required 42.33 cc. 0.1N hydrochloric acid.
- III. 0.8000 gram required 43.98 cc. 0.1N hydrochloric acid.

NO.	NITROGEN FOUND	THEORETICAL NITROGEN
	<i>per cent</i>	<i>per cent</i>
I	7.76	7.73
II	7.81	7.73
III	7.71	7.73

³ "Casein from milk, washed," obtained from Eimer and Amend, was used in the experiments.

A STUDY OF THE EFFECT OF CHANGES IN THE CIRCULATION OF THE LIVER ON NITRO- GEN METABOLISM.

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INTRODUCTION.

Hahn, Massen, Nencki and Pawlow¹ were among the first to undertake any very extensive metabolic experiments upon animals with the blood-supply to the liver more or less interfered with. In addition to cutting off the portal blood to the liver by means of an Eck's fistula, they ligated the hepatic artery and even went so far as to try to extirpate the liver altogether so as to totally remove its influence. The latter they accomplished only in part for they always left behind about 15 to 20 per cent of the total mass of the organ. However the partial extirpation of the liver in no way changed the results obtained by ligating the hepatic artery after an Eck's fistula; in either case the animals only survived the operation for from ten to twenty hours. It is quite evident, from their experiments, that the condition of the animal during the short lease of life left it after the operation, rendered it impossible to obtain any trustworthy information concerning what might have been the changes in metabolism had the conditions imposed by the operation permitted the animal to live.

During the course of their experiments they found that portal blood might contain, especially during protein digestion, three to five times as much ammonia as the blood of the general circulation; but after the establishment of an Eck's fistula, while they were unable to find any appreciable increase in the ammonia con-

¹ Hahn, Massen, Nencki, Pawlow: *Arch. f. exp. Path. u. Pharm.*, xxxii, p. 161, 1893.

tent of blood of the general circulation, the proportion of ammonia nitrogen to total nitrogen and urea in the urine was increased. They also found in their experiments, when in addition to an Eck's fistula they ligated the hepatic artery and cut away a large part (85 per cent) of the liver tissue, the ammonia not only increased in the urine, at the expense of the urea, but the ammonia content of the systemic blood actually increased, even to a point equal to that present in the portal blood, and gave rise to symptoms similar to those of ammonia poisoning, which proved fatal.

Perhaps the most interesting observation made by these investigators, as well as a most stimulating one, was, that in some of the Eck's fistula dogs, the ingestion of meat was followed sooner or later by a train of symptoms of a nervous character, similar, in some respects at least, to the symptoms of ammonia poisoning. These observations led to a number of subsequent investigations by these same authors as well as by others, viz.: Biedl and Winterberger,² Rothberger and Winterberger,³ Salaskin,⁴ Nencki and Zaleski,⁵ and later by Macleod,⁶ Hawk,⁷ Fischler,⁸ Grafe and Fischler,⁹ all of whom dealt more or less with the relationship of ammonia to these toxic symptoms. While all agreed as to the symptoms and the increase in the ammonia content of the blood, yet all (except Salaskin) were unconvinced that ammonium carbonate, or ammonium carbamate, or in fact any other ammonia compound or NH_2 compound upon which the liver is capable of acting, was the sole cause of the intoxication. Salaskin however was fully convinced that the symptoms were entirely due to ammonia.

The question to which attention was first directed in these investigations was the fate of the products of intestinal digestion and absorption which under normal conditions pass into the portal blood to be carried to the liver, before entering the general circulation. Of the nitrogen compounds formed in the intestines, am-

² Biedl and Winterberger: *Arch. f. d. ges. Physiol.*, lxxxviii, p. 140, 1902.

³ Rothberger and Winterberger: *Arch. internat. de physiol.*, ii, pp. 140-141, 1905.

⁴ Salaskin: *Zeitschr. f. physiol. Chem.*, xxv, pp. 128, 449, 1898.

⁵ Nencki and Zaleski: *ibid.*, xxx, p. 193, 1901.

⁶ Macleod and Haskins: *this Journal*, i, p. 319, 1905.

⁷ Hawk: *Amer. Journ. of Physiol.*, xxi, p. 259.

⁸ Fischler: *Deutsch. Arch. f. klin. Med.*, ciii, p. 156, 1911; *ibid.*, civ, p. 300.

⁹ Grafe and Fischler: *ibid.*, civ, p. 319, 1911.

monia was the one to which special attention was directed, and that because it was always present, and was about the only one known to possess any very marked toxic properties. Also the action of the liver upon ammonia formed in the intestines was quite well known.

EXPERIMENTAL.

I. Methods used for the determination of ammonia contained in the blood and their comparative accuracy.

The methods heretofore employed for the estimation of small amounts of ammonia such as are present in the fluids of the body were not very accurate, although, in the hands of some, they led to quite accurate results as compared with the results obtained by the more modern methods. However the published results show such wide variations, only to be accounted for by the inaccuracy of the methods used, that we deemed a reinvestigation necessary. Hence it was of the utmost importance to ascertain that the method to be employed here was sufficiently accurate to come well within the limits of the small amounts of ammonia which are involved.

Hitherto the determinations of ammonia in the blood (Folin's later work excepted) were made with quantities of blood (25 to 50 grams) which at most would not contain more than 0.5 mgm. and generally not more than 0.1 to 0.2 mgm. This after being carried over into an $\frac{N}{10}$ or $\frac{N}{100}$ H_2SO_4 solution was titrated, using some one of the indicators, generally alizarin red, to determine the end-point. It is well known that most indicators do not react with the greatest precision and therefore are not applicable when very small amounts are to be taken into account. This is especially true with ammonia, which is likely to form hydrolyzed salts with the indicator and thus give an indefinite end-point. In fact when not more than 0.5 mgm. of ammonia is absorbed in 100 cc. of $\frac{N}{10}$ H_2SO_4 solution, it is impossible to titrate with sufficient accuracy to even approximate the actual amount, the limits of error being several times greater than the amount of ammonia to be determined.

The method employed by us in all of the experiments was the well-known Nessler's method. This method is known to be accurate enough to distinguish 0.01 mgm. of ammonia and is therefore at least ten times more accurate than the titration methods heretofore ordinarily used. In fact it is the only known method accurate enough to determine quantities of ammonia ranging from 0.01 to 0.05 mgm.

Preliminary controls with known amounts of ammonia showed that we were always able by this colorimetric method, to recover at least 98 per cent of the known amount of ammonia present, using amounts of not more than 0.2 mgm. in each test.

In making the estimations of ammonia in blood, we used the following procedure:

Sufficient blood was drawn from a dog's heart to make 50 grams of defibrinated blood, of which 25 cc. were put into each of two aëration cylinders (Folin), *A* and *B*. To *A* were added 5 grams of sodium chloride plus sodium carbonate sufficient to render the blood strongly alkaline, after which it was subjected to a strong current of air for six hours according to the method of Folin. To cylinder *B* was added sufficient ammonium chloride to liberate 0.5 mgm. of NH_3 and then treated exactly as *A*. If cylinder *A* showed 0.35 mgm., cylinder *B* should show 0.35 mgm. plus the 0.5 mgm. added or 0.85 mgm. Unless tube *B* estimated within 10 per cent of this theoretical amount the results were rejected. However this became necessary only three or four times.

The comparative results of the colorimetric and the titration methods are shown in table I.

TABLE I.

Comparative results of titration and Nessler methods on normal dogs.

NUMBER OF DOG	MGMS. OF NH_3 PER 100 GMS. OF BLOOD	METHOD EMPLOYED	DIET
1	1.16	Titration	Street
2	1.78	Titration	Street
3	1.17	Titration	Street
4	1.58	Titration	Street
5	1.39	Titration	Street
Average.....	1.42		
6	0.42	Nessler	Street
7	0.43	Nessler	Street
8	0.625	Nessler	Street
9	0.59	Nessler	Street
10	0.32	Nessler	Street
11	0.32	Nessler	Bread and milk
12	0.35	Nessler	Bread and milk
13	0.42	Nessler	Bread and milk
14	0.30	Nessler	Bread and milk
15	0.37	Nessler	Carbohydrate
Average.....	0.416		

The above results obtained by the titration method agree very well with those obtained by other workers, by similar methods, but represent about three to four times the actual amount of ammonia present, as shown by the Nessler method. However it must be stated that certain other investigators, especially Horodyski, Salaskin and Zaleski¹⁰ obtained results agreeing very closely with the above (Nessler's method). Their tables show an average ammonia content for arterial blood (dogs) of 0.41 mgm. per 100 grams; for portal blood (fasting), 1.29 mgm., and for portal blood during protein digestion of 1.85 mgm.

Folin,¹¹ working with cat's blood, employing the calorimetric method and using small quantities of blood drawn into weighing bottles containing sufficient potassium oxalate to prevent coagulation, recovered extraordinary small amounts of ammonia, only traces in the arterial blood and about 0.15 mgm. per 100 grams in the portal blood. He aerated the blood for a short time, not more than thirty minutes, thinking that a longer period might give rise to decomposition ammonia. With dog's blood we were unable to confirm his findings, the method given for arterial blood 0.35 mgm. per 100 grams and for portal blood (same dog during digestion), 0.68 mgm.

TABLE II.

Comparison of the ammonia content of the portal blood with that of the common carotid of the normal dog.

DOG NO.	KIND OF BLOOD	MGM. OF NH_3 PER 100 CC. OF BLOOD	METHOD
I	{ Portal.....	0.800	Nessler
	{ Arterial.....	0.420	Nessler
II	{ Portal.....	0.860	Nessler
	{ Arterial.....	0.430	Nessler
III	{ Portal.....	1.220	Nessler
	{ Arterial.....	0.625	Nessler

These dogs were examined at the height of protein digestion, about five hours after feeding 1.5 pounds of lean meat each. The results agree in general with those of other investigators and leave

¹⁰ Horodyski, S; Salaskin and J. Zaleski: *Zeitschr. f. physiol. Chem.*, xxxv, p. 246, 1902.

¹¹ Folin and Denis: *this Journal*, xi, p. 161, 1912.

no doubt but that ordinarily the portal blood carries about twice as much ammonia as the systemic blood. Although not shown in the above tables our analyses were confirmatory of the findings of previous workers, that the blood of the hepatic veins contains no more ammonia than is present in the arterial blood, thus proving that the excess ammonia contained in the portal blood is taken out during its passage through the liver.

Repeated examinations of the systemic blood of individual dogs showed that the ammonia content was practically constant for each animal examined, irrespective of the diet, state of digestion or condition of the bowel. While repeated examinations of the portal blood of the same dog are not permissible, dogs examined under different conditions showed a variation in the ammonia content of the portal blood. Blood taken during high protein digestion or when the bowels were full, especially when much fecal matter was present, always gave a high ammonia content as compared with fasting animals, or after the lower bowel had been cleansed by purgation. Just what proportion of the ammonia formed in the intestines as the result of protein digestion is difficult to estimate; at least a good percentage of it is given off from the feces in the lower bowel (Folin).¹²

II. Eck's fistula dogs.

Out of a total of thirty-five dogs with Eck's fistula, only three responded to meat feeding with the typical symptoms described in the literature. One of these we wish to speak of more or less in detail.

Dog I. This dog, a large female weighing 12 kgm., was killed when in good health fifteen months after an Eck's fistula operation and had suffered seven attacks of meatintoxication. Following the operation, she was kept on a bread and milk diet for thirty days, during which period she lost some flesh, but otherwise was apparently normal. She was then put on a generous allowance of raw lean beef which she ate ravenously, seeming to enjoy the change. She gradually became lively and restless and in about ten days was showing symptoms, simulating the restlessness and irritability so characteristic in mild cocaine intoxication. These

¹² *Loc. cit.*

symptoms increased up to the fifteenth day of the meat feeding when the dog became delirious and during the following day suffered frequent attacks of clonic convulsions. All food was refused, but warm milk was administered through a stomach-tube. After the first milk feeding, recovery began to take place and the animal was normal in forty-eight hours.

The symptoms, as they developed, suggested a gradually increasing cerebral stimulation, beginning first with the highest areas (caffein-like), followed by a like action upon the motor areas (cocaine-like), and passing on to the medulla, so that

TABLE III.

Ammonia in the blood of Dogs 1 and 25, before operation; after operation; periods of good health; and periods of meat intoxication.

Dog 1.

DATE	NH ₃ IN MG. PER 100 CC. BLOOD	DIET	REMARKS
1910			
Feb. 10	0.4	Mixed	Eck's fistula performed.
July 14	0.42	Bread and milk, 15 days	Dog in good condition.
July 20	0.52	Meat, 5 days	Blood drawn at height of digestion.
July 26	0.6	Meat, 11 days	Dog very irritable, active, in delirium.
July 27	0.52	Eaten nothing for 36 hours.	Clonic convulsions.
Aug. 2	0.49	Bread and milk, 6 days	Dog in good condition.
Aug. 11	0.53	Meat and Liebig's ext. (2 oz. per day) for 5 days.	No toxic symptoms.
Aug. 17	0.66	Meat and Liebig's ext., 15 days	Typical toxic symptoms; blood-pressure low, and blood dark-colored.
Nov. 14	0.49	Bread and milk, 30 days	Dog in good condition.
Nov. 18	0.48	Bread and milk, 30 days	Dog in good condition.
1911			
May 12	0.43	Mixed	Killed.

Autopsy. Abdominal viscera free from adhesions or other evidences of collateral circulation. The liver was small and had undergone fatty necrosis almost to complete destruction. Kidneys normal. Ratio of ammonia nitrogen to urea nitrogen, 1:32.

TABLE III—Continued

Dog 25.

DATE	NH ₃ IN MG. PER 100 CC. BLOOD	DIET	REMARKS
1911 Apr. 29	0.215	No food for 24 hours	Just before Eck's fistula operation. 9 a.m.
May 1	0.376	Cooked meat	Operation at 10.30.
May 9	0.54	Cooked meat (not eaten)	Blood drawn 1 hr. after meat feeding.
May 9	0.502	Meat (not eaten)	Dog had eaten nothing for 12 hours; very weak, although no toxic symptoms present. 9.30 a.m.
May 23	0.38	Mixed diet since May 9	Marked toxic symptoms; convulsions—clonic upon tonic. 10.30 a.m.
June 2	0.56	Meat diet from May 23	Dog in good condition.
June 12	0.5	Mixed diet	Dog in hypersensitive condition, bordering on convulsions.
June 14			Dog improved but still noisy. Killed.

Autopsy. Abdominal cavity perfectly clear. Fistula was wide open. No adhesions.

the final stage gave a very mixed set of symptoms, hard to define pharmacologically.

It is worthy to note that it required about twelve to fifteen days of intensive meat feeding to bring on the intoxication. An ordinary mixed diet was not sufficient. As stated this dog was subjected to intensive meat feeding seven times, and was just as susceptible to the intoxication fifteen months after the operation as one month—no tolerance nor immunity having been established.

There was no appreciable increase in the ammonia content of the blood following the fistula operation, it being about 0.4 mgm. per 100 grams before and after the operation; but the proportion of the ammonia nitrogen to the total nitrogen and urea in the urine was increased, almost wholly at the expense of the urea. This disarrangement of the nitrogen constituents of the urine almost wholly disappeared after eight to ten months. As shown in table III, the ammonia content of the blood was somewhat greater

during the periods of meat feeding, especially at the beginning of the acute symptoms, as was also the ammonia in the urine. During the acute intoxication the urine was diminished not more than 50 to 75 cc. per 24 hours as compared with 250 to 300 cc. when on a mixed non-toxic diet.

As a further study of the toxic action of the portal blood when saturated with the products of protein digestion, three dogs were operated on five hours after the ingestion of 2 pounds each of Hamburger steak. These animals only partially recovered from the effects of the operation. In two or three hours they were walking about, but gradually lapsed into a comatose condition and died in from ten to twenty-four hours. At autopsy, the fistulae were found wide open and no congestion or stasis of the portal circulation was present.

To compare with these, dogs were operated on three hours after the ingestion of 300 cc. of milk, containing 50 grams of starch, 50 grams of glucose and 30 cc. of olive oil. These dogs recovered as promptly as did the dogs which were operated on after one day of fasting. This is taken as an indication that when the portal blood, taken at the full tide of protein digestion, is suddenly turned into the general circulation, it may produce profound depression, coma and death; whereas if time is given for the adaptation of the body to the Eck-fistula before the meat feeding, as was usually done, there was no such profound depression. However, other experiments showed that it took only a short time for the animals to become adapted to the new condition. Several dogs were operated on after having fasted for forty-eight hours and meat feeding was instituted twenty-four hours afterwards. These dogs seemed no more susceptible to meat poisoning than when the meat feeding was instituted thirty days after the operation. In fact, none of these dogs proved susceptible.

III. The effects of Eck's fistula and ligation of the hepatic artery.

1. *Eck's fistula and hepatic artery ligated at the same time.* Dogs so operated upon recovered from the immediate effects of the operation but as a rule, after from eight to ten hours, they began to lapse into a comatose condition and died within twenty-four hours. Some of the animals did not live for more than twelve hours, in

fact these dogs behaved very much like those on which the Eck's fistula was made at the height of protein digestion. Autopsies made immediately following death showed the liver undergoing rapid autolysis and, in those cases where the animal lived for twenty-four hours, the whole organ was in a state of disintegration emitting a fatty acid odor. In fact, some gas was free in the abdominal cavity and generally from 25 to 50 cc. of dark-colored fluid. Blood drawn during the coma showed a marked increase in the ammonia content which was equal to that found in blood immediately following death.

2. *Hepatic artery ligated several weeks after Eck's fistula operation.* In four dogs so operated upon a somewhat different state of affairs resulted as shown in table IV. One of these (No. 14) had the hepatic artery ligated on April 18 and lived until the morning of April 21, when life was terminated on account of an accident. This animal ate meat and passed about 300 cc. of urine. At death 25 cc. of urine were taken from the bladder which contained 4.5 per cent total nitrogen of which about 60 per cent was urea and 20 per cent ammonia. Autopsy showed: no adhesion, the hepatic artery ligated, the fistula open 1 cm. in diameter, and the portal vein completely closed. The liver was small and firm with a few darkened areas scattered over the surface. Had not life been terminated by accident, everything indicated that the animal would have lived for several days longer.

The other three dogs died within thirty hours, one only lived for nine hours and died while in a state of strychnine-like convulsions. The other two (23 and 25) which lived for twenty-four hours or more, recovered from the immediate effects of the operation, ate meat and drank water. About fifteen hours after the operation, they began to show signs of hypersensitiveness which developed into convulsions, both clonic and tonic in character, in which condition they died.

The autopsies showed: no peritonitis, no fluid in the abdominal cavity, the hepatic artery and the portal vein completely shut off, and the fistulae were open and about 1 cm. in diameter. The livers were well preserved, not having undergone any marked acute autolysis. Adhesions were quite extensive especially in the lower surface of the liver, apparently sufficient to furnish a fair supply of blood to the organ.

These dogs, although their livers showed no acute autolytic changes as did the livers of the dogs upon which both operations had been made at the same time, lived, with the exception of dog 14, only a few hours longer than the latter. Only a few cubic centimeters of urine were excreted, highly acid, and as shown in table IV the ammonia content of the blood had increased to a point apparently equal to that necessary to cause poisoning.

IV. Dogs showing digestive disturbances.

Of the remaining dogs, ten ran rather a peculiar course. They recovered readily from the immediate effects of the operation, ate well of any food for which dogs have a liking, but in a few days began to lose weight and ran down in general health. They suffered more or less from diarrhoea, the feces being clay-colored with a fetid odor and contained an excess of fat. It was almost impossible to keep these animals even in a fair state of nutrition. Most of them went from bad to worse and died in from six to eight weeks in an extreme state of inanition. Autopsies on these animals showed the fistulae open, with practically no adhesions. The course run by these dogs was quite similar to that after the establishment of a permanent biliary fistula in which case the normal nutrition is hard to maintain. Also if the pylorus be closed and a gastro-enterostomy be made 40 cm. distal to the pylorus, dogs will suffer like digestive disturbances and as a rule die within two months from inanition. While quite foreign to the subject in hand, it might be stated in this connection that in some dogs, partial removal of the pituitary gland is followed by a like train of symptoms and death in about the same length of time. In seven of these dogs we could not make out any increased ammonia content of the blood. In three, however, the ammonia content of the blood was distinctly increased as shown by the following figures.

Before operation (mixed diet): 0.35, 0.42, 0.39 mgm. per 100 grams.

After operation (average of three estimations): 0.51, 0.75, 0.65 mgm. per 100 grams. The urine showed the same metabolic changes as occur in starving animals.

These figures are almost as high as those given in table II for the portal blood, and are as high as obtained from other dogs when in convulsions from meat intoxication.

TABLE IV.

Ammonia in the heart's blood of four dogs with Eck's fistula after subsequent ligation of the hepatic artery.

Dog 14.

DATE	NH ₃ IN MGM. PER 100 CC. BLOOD	DIET	REMARKS
1912			
Jan. 28	0.4	Fasting 24 hours	9 a.m.—10.30, operation.
Feb. 9	0.52	Meat + 32 gms. Liebig meat ext. per day since Jan. 30	Marked meat poisoning. 10 cc. of 10 per cent calcium lactate injected intraven- ously, after which dog be- came quiet and slept several hours. No more convul- sions, recovery complete in 48 hours.
Feb. 14	0.49	Cooked meat and milk	Dog in good condition.
Mar. 25	0.512	Meat + 64 gms. Liebig meat ext.	Dog very irritable, ill natured and very noisy.
Apr. 14	0.52	Same diet	Very noisy, continued rest- lessness but no convulsions.
Apr. 18			Hepatic artery ligated.
Apr. 19 a)	0.761	Cooked meat	At ten, blood was drawn and two estimations made.
Apr. 19 b)	0.747		Dog in good condition.
Apr. 20			Abdominal wound broke open and dog had to be killed while seemingly in good condition.
Apr. 21			

Autopsy. No infection; liver small and blackened in spots; hepatic artery ligated; no adhesions; fistula 1.5 cm. in length.

Dog 23.

1911			
Apr. 25	0.32	Fasting	Blood drawn 9 a.m. Eck's fis- tula performed at 10.30.
Apr. 28	0.40	Bread and milk	Dog in good condition.
May 16			Hepatic artery ligated at 10 a.m.
May 17	0.92	Bread and milk	10 a.m. Dog in good condi- tion.
May 17	2.47	Bread and milk	Dog in convulsions (4. p.m.) 4.45, died.

Autopsy. Hepatic artery ligated; liver small and undergoing rapid autolysis; fatty acid odor.

TABLE IV—Continued.

Dog 25.

DATE	NH ₃ IN MG. PER 100 CC. BLOOD	DIET	REMARKS
1911 June 12	0.5	Mixed diet	Dog recovering from meat poisoning, hepatic artery ligated (Eck's fistula Apr. 29).
June 13	0.83	Milk (little taken)	Dog in good condition.
June 13 5 p.m.	0.96		Symptoms of cerebral excitations.
June 13 10 p.m.			Dog died in convulsions.

Dog 30.

Apr. 25	0.39	Fasting	Eck's fistula made.
June 2	0.55	Meat diet	Dog hypersensitive, very restless and noisy.
June 9	0.54	Meat diet	Hepatic artery ligated at 10 a.m.
June 9 5 p.m.	1.15		Convulsions.
June 9 7 p.m.			Died in opisthotonos.

V. Dogs—normal after operation.

The remaining dogs (22) showed neither the symptoms of meat poisoning nor the digestive disturbances of the group just described. They recovered quickly from the effects of the operation, ate heartily of meat, bread and milk or any other sort of food offered them. For a time they were lively, apparently hypersensitive, but in a few weeks settled down to a normal condition. As a rule they gained in weight. Autopsies showed very marked adhesions of the liver to the surrounding viscera with more or less stenosis of the fistula. In most cases, the liver was firmly adherent to the pancreas, duodenum, and to other parts of the intestine with which it came in contact. The adhesions were very vascular and appeared to be sufficient to take the place of the portal vein. In those

cases where the adhesions were most extensive, the stenosis of the fistula was almost complete. In one case the fistula was completely closed and even the main trunk of the portal vein was obliterated. Yet the animal lived and enjoyed good health.

The significance of such adhesion as a protection against meat poisoning and digestive disturbances has been pointed out by Rothberger and Winterberger.¹³ Under such conditions the liver apparently functions in the normal way, the blood going to the liver through the adhesions instead of the portal vein.

These findings corroborate the earlier findings of the St. Petersburg school already cited, namely, that an Eck's fistula does not necessarily cause an increase of ammonia in the systemic blood, but that in fistula-dogs susceptible to meat intoxication, ammonia does accumulate in the blood of the general circulation considerably above that normally present, and that after the ligation of the hepatic artery upon an Eck's fistula, the ammonia in the systemic blood may increase to an amount equal to or greater than that normally present in the portal blood, and that animals subjected to such operations soon die showing symptoms such as occur in acute ammonia poisoning.

DISCUSSION.

The change in the nitrogen metabolism most apparent under the conditions imposed by an Eck's fistula is that the proportion of ammonia nitrogen to urea nitrogen in the urine is increased without materially changing the total nitrogen output. Also some investigators have reported a temporary increase in the uric acid and lactic acid outputs, the latter being more lasting than the former. This suggests two possibilities; either the liver cannot act upon the nitrogen compounds contained in the portal blood, which under normal conditions it changes to urea, when they are carried to it in the arterial blood, or that these same nitrogen compounds are excreted so rapidly by the kidneys that only a minimum ever reach the liver.

Evidence, largely indirect, is not wanting that the former is the correct explanation. After an Eck-fistula, the liver ceases to store up glycogen in the usual quantities. The liver of such animals, even when well fed upon carbohydrate, corresponds in glycogen

¹³ Rothberger and Winterberger: *loc. cit.*

content to the liver of fasting animals, while the muscles may contain quantities of glycogen corresponding to those found in well-fed animals (De Filippi).¹⁴ In other words, the liver ceases to participate in the formation of glycogen, although, unlike the ammonium compounds, the sugar is not excreted in the urine but evidently circulates sufficiently long in the blood to come intimately in contact with the liver tissue as such contact takes place with the arterial blood. An Eck's fistula seems to influence very little the ability of an animal to utilize sugar, and it can hardly be said to lessen the sugar tolerance at all.

The effect of an Eck's fistula upon the formation of bile, might be cited as additional evidence that the liver does not act upon the precursors of bile when deprived of the portal blood. As showed by Voegtlin and Bernheim¹⁵ and corroborated by us, ligation of the common bile duct after an Eck's fistula induces no jaundice nor any accumulation of bile in the blood. This observation suggests one of two possibilities; either the liver forms bile from the constituents of the portal blood only, or the portal blood contains some substance which stimulates the liver to bile formation. This is contrary to the generally accepted view that the liver forms bile from the blood irrespective of its source.

Have we any evidence that a like condition obtains in regard to the formation of urea by the liver? It may be taken as proven that the quantities of ammonia in the portal blood above those present in the systemic blood are converted into urea by the liver and are not permitted to enter the general circulation at all; and that they constitute at least one of the sources of urea. It is also quite evident that when the portal-blood ammonia is diverted into the general circulation, and permitted to come in contact with the liver tissue by way of the hepatic artery only, it no longer serves as a source of urea but is eliminated in the urine as such. When we take into consideration that the kidney epithelium is very readily permeable to ammonia compounds in general, the above argument loses some of its force, for the kidneys, coming, as they would, in competition with the liver in ridding the blood of any excess of ammonia, might place the liver at a very great disadvantage.

In our series of experiments, dog 14 (table IV) furnished some information on this question. As already stated this dog, which

¹⁴ F. de Filippi: *Zeitschr. f. Biol.*, xlix, p. 511, 1907; I, p. 38, 1908.

¹⁵ Voegtlin and Bernheim: *Journ. of Pharm. and Exp. Ther.*, ii, p. 55, 1911.

was susceptible to meat intoxication, had the hepatic artery ligated several weeks after the Eck's fistula was established and lived in good condition for forty-eight hours afterwards with every indication that life would have continued longer, had it not been terminated by accident. The animal drank water and ate meat during the short lease of life after the last operation and passed 300 cc. of urine. At death the stomach contained food, and the urine (25 cc.) which was taken out of the bladder when killed, contained 4.5 per cent total nitrogen of which about 60 per cent was urea and 20 per cent ammonia nitrogen. We could find no evidence that, after the ligation of the hepatic artery the liver had received any blood by way of the portal vein or hepatic artery, both being completely closed. This, while it does not bring forward any conclusive evidence that the liver does not form urea from urea precursors when they are presented to it in the arterial blood, does furnish evidence, at least, that urea may be formed by tissues other than those of the liver. But the fact that the ligation of the hepatic artery did not materially add to the disarrangement of the nitrogen constituents in the urine occasioned by the Eck's fistula, argues that the liver does not form urea from ammonium salts when circulating in the systemic blood alone.

This together with the evidence furnished by dog 1 (table I), which lived fifteen months with an Eck's fistula, was subjected to meat intoxication seven times, and the liver of which had undergone degeneration almost to complete destruction, yet excreted, during the last two months of life, urea up to 80 per cent of the total nitrogen, while for some weeks following the operation the urea nitrogen was only 60-65 per cent of the total nitrogen excreted, suggested the following speculation: The liver not only does not form urea from ammonia or ammonium compounds when presented to it in the arterial blood, but that, when the liver ceases to be a factor in the formation of urea, the other tissues of the body gradually assume the work in this regard, normally done by the liver. This is paramount to saying; that no specific tissue of the body enjoys the sole monopoly to form urea in the process of nitrogen metabolism, but that urea is formed by the tissues in general, at least primarily; and that after the removal of certain specialized tissues (organs) which possess this power to a greater degree than the general tissue, there follows a reversion to the original form of metabolism by the tissues in general which may be sufficient to

maintain the nutrition of the body without the intervention of specialized tissue.

While these changes in metabolism occur after an Eck-fistula, they are not wholly characteristic of the conditions of life imposed by the operation. Ligation of the hepatic artery, alone, brings about the same changes as does an Eck's fistula, *i.e.*, the ammonia compounds in the portal blood, which normally are changed into urea by the liver, are no longer changed but pass into the general circulation to be excreted in the urine as such (Doyon and Dufourt).¹⁶ Phosphorus poisoning, with its characteristic changes in the liver cells, brings about a similar condition in metabolism, *i.e.*, ammonia is not changed to urea and oxidizable acids may appear in the urine.

It is well known that after an Eck's fistula the liver soon begins to undergo fatty necrosis which eventually invades the whole organ. This change in the nutrition of the organ may be responsible, in large measure, for the changes in metabolism so noticeable after an Eck's fistula. If so, then the fact that the liver does not perform its chemical work when shunted out of the portal circulation would have no bearing upon the question, whether or not the liver acts chemically upon substances circulating in the systemic blood only.

Ligation of the hepatic artery is followed by no such nutritive changes in the liver, therefore the suspension of chemical work occasioned by ligation of the artery must be due to a diminution in the oxygen supply as suggested by Doyon and Dufourt already cited.

From what has been said, it seems that the exclusion from the liver, by way of the portal vein, of the products of protein digestion and absorption as they appear in the portal blood and the excretion of at least some of the precursors of urea (ammonia) in the urine is not incompatible with life and good health. Even when long continued, the augmented excretion of ammonia in the urine is without injurious effects on the kidneys (dog 1).

However, fistula dogs are susceptible to meat intoxication, while animals with the hepatic artery ligated are not. This then might be taken as evidence that the ammonium compounds absorbed

¹⁶ Doyon and Dufourt: *Compt. rend. de l'Acad. des Sci.*, 1898, p. 419; *Arch. de physiol. norm. et path.*, x, pp. 522-37, 1898.

from the intestines, and which are convertible to urea by the liver, are not the toxic agents in meat intoxication.

While we have attempted to reduce to a minimum the influence of the liver on protein and carbohydrate metabolism, we cannot regard it as unimportant in certain other respects. It evidently has the power to destroy certain substances formed from proteins during intestinal digestion and absorption, which are poisonous when not allowed to enter the portal circulation. This might be taken as further proof that the liver acts upon substances only when circulated through it in the portal blood. Again, the liver cannot be shunted out of the portal circulation without, in at least a good percentage of cases, affecting the digestion. As already stated, ten of our dogs died of inanition in from four to six weeks after the operation. The symptoms exhibited were quite similar to those following the establishment of a permanent biliary fistula, and even more so to the symptoms occurring after the pylorus is closed and a long looped (40-45 cm.) gastro-enterostomy made. In the latter case, dogs always die from inanition in from five to eight weeks, presumably from some interference with the formation of the digestive juices in the duodenum, probably due to the lack of the formation of "secretin." It might be imagined that an Eck's fistula, interfering as it does with the formation of bile, would bring about a condition similar to that following a permanent biliary fistula, or we might conceive, as has been suggested by others, that the liver forms some substance of the nature of an internal secretion, the formation of which is interfered with by an Eck's fistula. That the former will not suffice to explain the occurrence of digestive disturbances is shown by the following observation: Dogs having both an Eck's fistula and the common bile duct ligated may and often do return to a normal state of health, especially if extensive adhesions form about the liver, although no bile is permitted to enter the duodenum. This is rather paradoxical. After the formation of adhesions, we would expect the resumption of the formation of bile due to the fact that much of the return flow of blood from the intestines now circulates through the liver. There seems to be no resumption in the formation of bile, neither are there any digestive disturbances. This indicates that the adhesions bring about a partial return flow of portal blood which stimulates the liver to form some substance which offsets the deleterious effects occurring when no adhesions are formed.

ON FAT ABSORPTION.

II. ABSORPTION OF FAT-LIKE SUBSTANCES OTHER THAN FATS.

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The manner in which fats are absorbed is still largely a matter of opinion rather than of proven fact. Aside from the possibility of absorption in other channels than the chyle, it is a question whether saponification is a necessary preliminary to absorption, or whether some of the fat may be carried into the chyle intact either as an emulsion or in some sort of solution. It is generally recognized that under ordinary conditions of feeding most if not all of the fat which enters the blood through the chyle has been broken down and rebuilt by the intestine, but there is much evidence to show that under other conditions, especially in the feeding of large amounts of fat, food fats may be transferred to the fat depots of the animal body without marked change.

Dyes which are soluble in fats and fatty acids but not in aqueous soap solutions, have been shown to be absorbed in quantity.¹ Even bacteria have been shown to be aided in their passage through the intestinal wall by fats.² It is reasonable therefore to suppose that any substance which is soluble in fats and fatty acids might similarly be carried through the intestinal wall and that therefore at times fats may be absorbed in this way.

It is not possible to test this point with fats themselves, since fat so transported could not be distinguished from the synthesized fat of the chyle. Three classes of substances which have been used for this purpose are: (a) the readily saponifiable esters of the fatty acids other than triglycerides, (b) difficultly saponi-

¹ Mendel and Daniels: this *Journal*, xiii, p. 71, 1912.

² Ravenel: *Journ. of Med. Res.*, x, p. 460.

fiable esters of the fatty acids (cholesterin, etc., esters of wool-fat), and (c) the petroleum hydrocarbons.

The readily saponifiable fatty acid esters. Munk and Rosenstein,³ working with a case of human chyle fistula and also with dogs, fed cetyl palmitate and amyl oleate and demonstrated that none of these substances appeared in the chyle fat. Frank,⁴ after feeding dogs with ethyl esters of various fatty acids, found in the chyle the triglycerides of the fatty acids, but no trace of alcohol or its esters. Bloor⁵ fed to dogs highly optically active iso-mannid esters of the fatty acids and could find no optically active fatty substance in the chyle. Frank and Argyris,⁶ in similar experiments with the monoglycerides of the fatty acids derived from lard, found that the chyle fat contained only triglycerides. The saponifiable esters of the fatty acids are thus well absorbed and, as none of the unaltered esters appeared in the chyle, saponification would appear to be a necessary preliminary to absorption; although the reason for their absence from the chyle might also be that they merely did not escape the excellent facilities for hydrolysis in the digestive tract.

Unsaponifiable fatty acid esters. The cholesterin and iso-cholesterin esters which are the main constituents of wool-fat are very resistant to lipases, and since they form extremely fine emulsions with water, they would seem to be very suitable substances for testing the absorption of fat-like substances in other than water-soluble form. The feeding experiments of Connstein⁷ with these substances indicate, however, that they are not absorbed.

The petroleum hydrocarbons. The petroleum hydrocarbons are soluble in fat and fatty acids and when so dissolved, emulsify well with dilute alkalies, but cannot be reduced to water-soluble form in the intestine. In a series of experiments on rats with an emulsifiable mixture of one of these substances (vaseline) and lard, Henriques and Hansen⁸ found that the hydrocarbon could be quantitatively recovered from the feces and concluded that

³ Munk and Rosenstein: *Virchow's Archiv*, cxxiii, pp. 230, 484.

⁴ Frank: *Zeitschr. f. Biol.*, xxxvi, p. 568.

⁵ Bloor: this *Journal*, xi, p. 429, 1912.

⁶ Argyris and Frank: *Zeitschr. f. Biol.*, lix, p. 143.

⁷ Connstein: *Arch. f. (Anat. u.) Physiol.*, 1899, p. 30.

⁸ Henriques and Hansen: *Centralbl. f. Physiol.*, xiv, p. 313, 1900.

there could be no absorption of fat in other than water-soluble form. Bradley,⁹ after feeding an *emulsified* mixture of another hydrocarbon (liquid albolene) and olive oil, found that the chyle fat consisted of about equal parts of the two substances, and came to the opposite conclusion—that fats may be absorbed as emulsions.

The results of the work, cited above, as a whole, go to show that only those substances were absorbed which could be reduced to water-soluble form. In view of the contradictory nature of the evidence on the petroleum hydrocarbons and the meagre data with respect to the unsaponifiable esters it seemed desirable to make a more detailed examination regarding the absorption of these two classes of substances.

I. THE ABSORPTION OF HYDROCARBON OILS.

The hydrocarbon oils used were those sold under the trade names of "liquid albolene" and "white vaseline." They were fed alone and in emulsified and unemulsified solutions in olive oil or cocoanut oil.

A. Feeding experiments with unemulsified (but emulsifiable) mixtures of the petroleum hydrocarbons.

First, feeding experiments were conducted in which solutions of the unemulsified hydrocarbon oils were fed to cats and the amount of unabsorbed oils determined by extraction of the feces. For this purpose they were dissolved in equal parts by weight of a fatty oil and fed together with a sufficient ration of boiled lean meat freed from visible fat. Olive oil and cocoanut oil were chosen as solvents because they among the common oils represented as wide a variation in composition as could be obtained. Olive oil consists almost entirely of triolein, while cocoanut oil contains little olein, but large amounts of the saturated fatty acids (mainly lauric and myristic). Any difference in absorption due to difference in composition of the solvent oil would thus be noticed. The olive and cocoanut oils contained, as a rule, enough free fatty acid so that the solution emulsified well with dilute sodium carbonate. In those cases where it did not, enough oleic acid was added to produce the desired effect. The hydrocarbon oil solution was

⁹ Bradley: *Proc. of the Amer. Soc. of Biol. Chem.*, Baltimore, 1911.

liquid at room temperature, the vaseline solution melted at about 30°C.

The experiments lasted six days each. The seventh day's feeding was the ordinary meat feeding together with bone ash, which served to separate the periods. Feces were collected daily. After the appearance of the bone-ash feces, the cages were scraped out and then rinsed with ether which was added to the extraction ether. The feces were mixed with acid alcohol and dried on a water bath. When dry they were powdered and extracted with ether in a Soxhlet extractor for twelve to sixteen hours.

The extracted fatty material was saponified with alcoholic alkali and the petroleum products separated with the unsaponifiable matter according to the method of Kumagawa-Suto. Mixtures of hydrocarbon oil with olive and cocoanut oils were carried through the processes of extraction and separation in order to determine the accuracy of the processes for these mixtures. Both the extraction and separation were found to be accurate.

Details and results of the individual experiments are given in the table below:

TABLE I.

EXPERIMENT NO.	MATERIAL FED	AMOUNT OF HYDROCARBON OIL FED	HYDROCARBON OIL RECOVERED FROM FECES	
			Grams	Per cent
		<i>grams</i>		
I	Hydrocarbon oil alone.....	17	15.9	93
II	Hydrocarbon oil alone.....	18	18.2	100
III	Hydrocarbon oil and olive oil	14.2	13.2	93
IV	Hydrocarbon oil and olive oil	22	20.2	92
V	Hydrocarbon oil and olive oil	22	20.0	91
VI	Hydrocarbon oil and cocoa-nut oil.....	16.5	14.5	88
VII	Hydrocarbon oil and cocoa-nut oil.....	18.6	16.9	85.5
VIII	Hydrocarbon jelly (vaseline) and olive oil.....	16.0	16.4	100
IX	Hydrocarbon jelly (vaseline) and olive oil.....	17.5	17.3	98.8

As may be seen from the table, the recovery of the hydrocarbon jelly (vaseline) was practically complete as was also that of the hydrocarbon oil when fed alone, while there was a slight discrepancy in the recovery of the hydrocarbon oil when fed in solu-

tion in olive and cocoanut oils which may have been due to absorption. Taking into account, however, the error of the experiment, the amount absorbed was probably negligible. The results with the hydrocarbon jelly bear out the findings of Henriques and Hansen¹⁰ who after feeding solutions of vaseline to rats, found all (95 per cent) of the vaseline in the feces.

Since special precautions were taken not only to have the hydrocarbon products in solution in a readily absorbable oil, but also to have the mixture of such a composition that it would emulsify readily with dilute sodium carbonate solutions, the possibility of absorption of these substances either in solution in fats or fatty acids or in the form of emulsions is shown to be extremely unlikely.

*B. Chyle experiments with unemulsified (but emulsifiable)
mixtures of the hydrocarbons.*

In continuation and extension of the feeding experiments a further series of experiments was conducted in which after feeding the same oil mixtures as before, the absorbed fat was collected by cannula from the thoracic duct and examined for absorbed hydrocarbons. The operations for the insertion of the cannula into the thoracic duct were done with aseptic precautions,¹¹ making

¹⁰ Henriques and Hansen: *loc. cit.*

¹¹ It was believed that the ordinary method for collecting the chyle with the animal under the influence of an anaesthetic does not give the best results. The fat content of the chyle diminishes continuously from the beginning of collection, and if the stomachs of the animals are examined after death they are found to be distended with gas and to contain much of the material fed, while the intestines are empty. Digestion and absorption apparently cease after the animal has been under the anaesthetic for a time. It was thought therefore that a better digestion and a consequent greater richness of the chyle would result if the chyle could be collected after allowing the animal to recover from the anaesthetic. Attempts were therefore made to devise some means whereby they could be confined for this purpose. The attempts were mainly unsuccessful and the animals had to be reanaesthetized. Occasionally in the struggling the cannula would be pulled out and if it could not be inserted again the chyle was collected by pipette as it filled the wound cavity. In Experiment III the animal was successfully confined by the use of slings and, as may be seen from the protocol of the experiment, there was an improvement in the fat content of the chyle (as judged by its color) beginning about an hour after recovery from the anaesthetic.

a small opening and avoiding unnecessary injury to the tissues. By the use of a well paraffined cannula of narrow lumen (2 mm. or less) clotting of the chyle in the tube was entirely prevented. After the operation the wound was closed by suture. In the experiments below (with the exception of Experiment III), the chyle was collected in the ordinary way with the animal in ether anaesthesia. After collection and measurement, the chyle was transferred to a separatory funnel, saturated with potassium sulphate and extracted with ether over night. The ether solution was separated and the clear lymph evaporated to dryness, powdered and extracted with warm ether. The ether extract from the dried chyle was added to the first, the combined extract washed with water and then evaporated to dryness. The chyle fat was saponified with alcoholic potash and the unsaponifiable matter (which would contain the hydrocarbon oils) separated according to the method of Kumagawa-Suto.

EXPERIMENT I. *Hydrocarbon oil and olive oil, equal parts by weight.* A dog, weight 13 kgms., in good condition, having fasted for 48 hours, was fed 25 grams of the mixture at 8.45 a.m. The operation was begun at 11.15. Chyle was collected from 12.10 to 6 p.m. 12.10-1.30, 55 cc., milk-white; 1.30-2.45, 40 cc., becoming thinner; 2.45-4.00, 20 cc., as above; 4.00-4.45, 20 cc., thin; 4.45-6.00, 30 cc., almost transparent. Total, 165 cc. The chyle was extracted in three parts: (a) the first 55 cc., (b) the 110 cc. following, and (c) the dried chyle from (a) and (b). Total fat, 1.2 grams or 0.73 per cent of the amount of chyle. The fatty acids from this chyle fat melted at 30-32°C.

The analytical data of this and the succeeding experiments are given in Table II below.

EXPERIMENT II. *Hydrocarbon oil and olive oil, equal parts by weight.* A dog, weight 9 kgms., lean and active, after fasting for 48 hours, was fed 25 grams of the oil mixture at 8.45 a.m. The operation was begun at 11.15 and the cannula was in the duct and collection begun at 12.15. The duct ruptured at 12.30 and from then until 5 p.m. the chyle was collected by pipette as it flowed into the wound cavity. The chyle fat was extracted and the unsaponifiable matter separated in the regular way.

EXPERIMENT III. *Vaseline and olive oil, equal parts by weight.* A dog, weight 9 kgms., plump and in good condition, was fasted for 48 hours and fed 16 grams of the mixture at 9 a.m. Collection of the chyle began at 12.30. At 1.40 the animal was suspended in slings and allowed to recover from the anaesthetic. By 2.40 the effects of the ether had passed off and there was an immediate improvement in the fat content of the chyle, which lasted for about two hours. Chyle was collected for five hours. 12.30-1.40, 25 cc., white and milky; 1.40-2.40, 13 cc., becoming clear; 2.40-3.40, again milky; 3.40-4.40, 15 cc., milky; 4.40-5.40, 12 cc., again becoming

clear. The animal was becoming very restless and the experiment was concluded. Total chyle, 80 cc. The fatty acids from this chyle fat were solid at room temperature.

The analytical data are given in Table II below.

EXPERIMENT IV. *Vaseline and olive oil, equal parts by weight.* A dog, weight 11 kgms., after the usual fasting period, was fed 25 grams of the mixture at 8.45 a.m. Chyle was collected from 1 p.m. till 5.45 p.m., then extracted as before. The fatty acids from the chyle were liquid at room temperature.

EXPERIMENT V. *Hydrocarbon oil and cocoanut oil, equal parts by weight.* A dog, weight 9.5 kgms., thin, after a 48 hours' fast, was fed 34 grams of the mixture at 8.45 a.m. Collection was begun at 11.45 a.m. and continued until 5.45 p.m. The chyle, which at first was very rich in fat, gradually became less rich until at the end of the experiment it was almost transparent. (This diminution in the fat content of the chyle has been found to be characteristic of animals kept under ether anaesthesia for several hours and indicates a cessation of digestion, which is confirmed by post-mortem examination. The stomach is found to be distended with gas and to contain undigested material while the intestines are empty.) 11.45-1.25, 30 cc., rich and creamy; 1.25-2.25, 18 cc., milky; 2.25-3.25, 15 cc.; 3.25-4.40, 16 cc.; 4.40-5.50, 10 cc. Total chyle, 90 cc.

The analytical data are given in Table II.

TABLE II.

Chyle experiments with mixtures of petroleum hydrocarbons and oils.

CHYLE		CHYLE FAT		UNSATURIFIABLE MATTER*		
Time	Volume cc.	Amounts grams	Per cent of chyle	Amounts gram	Per cent of chyle fat	M. P. °C.
EXPERIMENT I. <i>Hydrocarbon oil and olive oil fed.</i>						
12.30-1.30	55	0.5	1.1	0.032	6.4	112
1.30-6.00	110	0.4	0.55	0.03	7.5	
	Dried	0.3		0.03	10.0	
EXPERIMENT II. <i>Hydrocarbon oil and olive oil fed.</i>						
12.15-5.00	65	0.8	1.2	0.048	6.0	132
EXPERIMENT III. <i>Vaseline and olive oil fed.</i>						
12.30-5.40	80	1.6	2.0	0.05	3.2	110
EXPERIMENT IV. <i>Vaseline and olive oil fed.</i>						
1.00-5.45	75	0.5	0.7	0.025	5.0	112
EXPERIMENT V. <i>Hydrocarbon oil and cocoanut oil fed.</i>						
1.45-5.50	90	1.2	1.3	0.063	5.3	110

* The unsaponifiable matter was crystalline in all cases.

From the data given above it may be seen that the unsaponifiable fraction (which would have contained the hydrocarbon products if present) is small in amount—mostly less than 7 per cent—and hence falls within the limits for unsaponifiable matter in the fat of normal dog chyle.¹² It is crystalline, its melting point is high (110°C. or over) and it gives the tests for cholesterin, from which facts we may conclude that the unsaponifiable matter consists, as normally, mainly of cholesterin. There is therefore no appreciable absorption of the hydrocarbon oils into the chyle. The results are thus in agreement with the results of the feeding experiments already reported, but are contrary to those of Bradley¹³ who found after feeding an emulsified mixture of equal parts of hydrocarbon oil and the olive oil that the chyle fat contained both substances in about the same proportions as fed.

C. Feeding of emulsions of the petroleum oils and examination of the chyle fat.

In Bradley's experiments the petroleum oil was fed as an actual emulsion and although there is little reason to believe that such an emulsion would behave differently from the emulsion formed normally in the intestine, a third series of experiments was conducted in which thoroughly emulsified mixtures of hydrocarbon oil and olive oil were fed and the chyle fat examined for hydrocarbon oils as before. Lecithin and gum acacia were used as emulsifying agents because of the resistance of emulsions formed with them to the action of the gastric juice.

The lecithin emulsion was made by dissolving 5 grams of lecithin (Merek) in 50 cc. of water and adding 45 grams of a mixture of equal parts of hydrocarbon oil and olive oil in small portions with continuous shaking, the whole operation taking about one hour. The emulsion so prepared showed no signs of separation of the oil in three days.

The acacia emulsion was prepared according to directions in the *National Formulary* with the same oil mixture as above, and then diluted with water until it would readily pass through a stomach

¹² Munk: *Virchow's Archiv*, cxxiii, p. 230.

¹³ Bradley: *loc. cit.*

tube. The emulsion showed no signs of disintegration in the course of a week.

After a preliminary fasting period the dogs were fed with the emulsions and about two hours later the operation for insertion of the cannula into the thoracic duct was performed. A new procedure was adopted with the intention of securing a better and more natural flow of chyle. As soon as the operation was completed the anaesthetic was stopped, the wound closed by sutures, the animal removed to a well-padded table and secured on his side so that the chyle from the cannula would drop into a beaker. As soon as the effects of the anaesthetic had passed off the animals generally ceased struggling and frequently went to sleep. Water was given as soon as and as often as they would take it. An improvement in the fat content of the chyle was noted generally within two hours after placing on the table. Chyle was collected as long as convenient or until the dog became restless. Before removal to the cage a broad metal collar with a slot for the cannula was fitted on. The lymph was in most cases still flowing on the second day and occasionally a second experiment could then be done with the animal. The cannula generally came out on the third day, after which the wound healed and the dog in the course of a short time was none the worse for the experiment.

EXPERIMENT I. *Lecithin emulsion of equal parts of hydrocarbon oil and olive oil.* A dog, young and active, weight 7.5 kgms., after a preliminary fasting for 48 hours was fed 50 grams of the lecithin emulsion (containing 22.5 grams of the oil mixture) at 10 a.m. and operated on at 12.00 m. The cannula was in the duct and collection begun at 1 p.m. As soon as the cannula was secured in the duct the wound was closed by sutures and the animal removed to a padded table, covered warmly and allowed to recover from the anaesthetic. Water was given as desired. The chyle was collected in a beaker as it dropped from the cannula. At first it was almost transparent, but in the course of two or three hours, as the animal recovered from the anaesthetic, the fat content improved and continued good to the end of the period of collection. Chyle was collected for eight hours, during which time the animal rested quietly—much of the time asleep. 1-2 p.m., 40 cc., poor in fat; 2-3 p.m., 15 cc., poor in fat; 3-4 p.m., 10 cc., milky; 4-5 p.m., 14 cc., milk-white; 5-6 p.m., 30 cc., milk-white; 6-7 p.m., 22 cc., cream-like; 7-8 p.m., 22 cc., as before; 8-9 p.m., 26 cc., as before. Total chyle, 179 cc. Next morning the chyle was still flowing and was almost water-clear so another feeding of emulsion was given, but before

the time for collection the flow ceased. The chyle was extracted in two portions: (1) the first 65 cc., (2) the remainder (114 cc.), after which the two portions were united, evaporated to dryness and the dry material extracted. The extracted fat was saponified and the unsaponifiable matter separated as before. The fatty acids¹⁴ from this fat melted at 29.5°.

The analytical details of the experiment are given in the table (III) below.

EXPERIMENT II. *Acacia emulsion of hydrocarbon oil and olive oil.* A dog, old, but in good condition, was fasted for 48 hours, then at 8.45 a.m., fed an acacia emulsion of 35 grams of the above oil mixture. The operation for insertion of the cannula was performed as usual and the collection of the chyle begun at 12.15. The animal was made comfortable on the padded table and as soon as he had recovered from the anaesthetic was given water freely. This dog struggled considerably and the flow of chyle lasted only six hours. 12.15-1 p.m., 23 cc., rich in fat; 1-2, 15 cc., rich in fat; 2-3, 22 cc., milk-white; 3-4, 18 cc., fat content decreasing; 4-5, 14 cc., thin; 5-6.30, 8 cc., almost transparent. Total chyle, 100 cc. The fatty acids melted at 30°C.

The analytical details of the experiment are given in the table below.

TABLE III.

Chyle experiments with emulsions of the hydrocarbon oil.

CHYLE		CHYLE FAT		UNSAPONIFIABLE MATTER		
Time p.m.	Volume cc.	Amounts grams	Per cent of chyle	Amounts gram	Per cent of chyle fat	M. P. °C.
EXPERIMENT I. <i>Hydrocarbon oil and olive oil, lecithin emulsion.</i>						
1.00-4.00	65	0.3	0.47	0.095	1.7	80
4.00-9.00	114	4.6	4.0			
	Dried	1.0	3.3			

EXPERIMENT II. *Acacia Emulsion.*

12.15-6.30	100	4.1	4.1	0.086	2.1	120
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The fat absorption in these experiments was very good—52 per cent in 8 hours and 24 per cent in 6 hours¹⁵ respectively—indicating that the processes of saponification and absorption of the fats were proceeding in a nearly if not quite normal manner.

In these experiments, as in the preceding, the amount and nature of the unsaponifiable matter precludes the possibility of the ab-

¹⁴ A more detailed examination of this material is being made and will be reported in a later publication.

¹⁵ Actually, in four hours, since there was practically no fat in the chyle of the last hour and a half.

sorption of any appreciable amount of the hydrocarbon oil. The results, while contrary to those of Bradley, are in agreement with all other reported results with the petroleum hydrocarbons and give no grounds for the belief that these substances are absorbed from the intestine.

II. THE ABSORPTION OF WOOL-FAT.

The hydrocarbon oils are substances which are never normally present in the tissues of animals and probably cannot be utilized by them. Their failure of absorption may have been due to a protective mechanism acting to prevent the absorption of fat-like substances which cannot be utilized. Cholesterin and its fatty acid esters on the other hand are constant constituents of the blood and other tissues and are therefore presumably useful substances. If it be found, as the experiments of Connstein indicate, that these substances are not absorbed, their rejection would be on a different basis from that of the hydrocarbon oils. The cholesterin esters of wool-fat, while they form excellent and finely divided emulsions with water and dissolve in fats and fatty acids, are not saponified by the lipases of the intestinal tract. Consequently if absorption depended on water solubility with hydrolysis as a necessary preliminary, these substances would be rejected, while if there were any absorption as emulsions or in solution in fats, they would be absorbed.

In the following experiments wool-fat (Merek's Lanolin) was fed to cats together with an adequate diet of boiled lean meat, free from fat. In order to make sure that the melting point of the wool-fat as fed was well below body temperature, as well as to get the benefit of any solvent effect of ordinary fats or fatty acids during absorption, the wool-fat was fed in solution in an equal weight of olive oil.

The experiments lasted six days each with an extra day in which bone ash was fed with the meat instead of the wool-fat. At the end of each period the feces were dried, ground and extracted with ether in a Soxhlet extractor for sixteen hours. Since it was not possible to separate the wool-fat from the ordinary feces fat, a three weeks' fore period was conducted on the diet without the wool-fat, and the average fat excretion for one week for each

animal determined. This was taken as the basal fat excretion and the difference between the total fat excretion on the wool-fat diet and this basal fat excretion was considered as unabsorbed wool-fat. The experiments resulted as follows:

EXPERIMENT I. *Fed 16.8 grams of wool-fat.*

Total ether extract of feces..... 17.1 grams.

Basal fat excretion..... 1.3 grams.

Wool-fat recovered..... 15.8 grams, 94 per cent.

EXPERIMENT II. *Fed 17.8 grams of wool-fat.*

Total ether extract of feces..... 20.4 grams.

Basal fat excretion..... 2.1 grams.

Wool-fat recovered..... 18.3 grams, 100 per cent.

The results thus bear out those of Connstein and show that wool-fat is not absorbed from the intestine. Since these esters differ from ordinary fats and other absorbable esters chiefly in that they cannot be saponified by the intestinal lipases, their rejection is in all probability due to that fact.

SUMMARY AND CONCLUSIONS.

In the foregoing experiments the absorption of two classes of fat-like substances, petroleum hydrocarbons and unsaponifiable esters (wool-fat), has been investigated. None of them were found to be absorbed.

The substances as fed were similar to ordinary fats in most of their properties. They emulsified well with dilute alkalies, were soluble in fats and fat solvents, and melted below body temperature. They differed from the fats mainly in that they could not be reduced to water-soluble form in the intestine.

The slow passage of the fats from the stomach, the abundant provision for hydrolysis and for the absorption of the products of hydrolysis in the intestine and the failure of absorption of fat-like substances which cannot be changed to a water-soluble form, make it extremely probable that fats can be absorbed only in water-soluble form and that saponification is a necessary preliminary to absorption.

The significance of this mechanism is little understood but in the light of the above results, one of its uses would appear to be to exclude undesirable fat-like substances which would otherwise be carried in with the fats.

The operations for insertion of the cannula into the thoracic duct were performed by Dr. W. McK. Marriott of this laboratory, to whom I take this opportunity of expressing my indebtedness.

RESEARCHES ON PURINES. XI.¹
ON 2,8-DIOXY-6-METHYL-9-ETHYLPURINE.

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(Received for publication, May 19, 1913.)

Although a large number of purines containing methyl groups are known, but very few purines containing ethyl groups have been described. Sembritzki,² working according to the method of Fischer and Ach,³ prepared 1,3-diethylpseudouric acid from which he obtained 1,3-diethyluric acid. By means of the same method, Armstrong⁴ synthesized 9-ethylpseudouric acid (VIII) and from this compound he prepared 9-ethyluric acid (IX). He also found that the latter compound could be alkylated further by means of ethyl iodide and in this manner he obtained a diethyluric acid of unknown constitution.

The method of preparing the 9-ethylpurine derivative, which we describe in this communication, is similar to that already used in this laboratory for the preparation of 9-methylpurines,⁵ namely, to first obtain an orthodiaminopyrimidine in which a hydrogen atom of the amino group in position 6 (or 4) has been replaced by an ethyl group (V).

The reactions employed in the synthesis of 2,8-dioxy-6-methyl-9-ethylpurine (IV) are as follows: 2-Ethylmercapto-4-methyl-6-chloropyrimidine (I)⁶ was heated with aqueous ethylamine and gave an excellent yield of 2-ethylmercapto-4-methyl-6-ethylaminopyrimidine (II). The latter compound was then boiled with hydrochloric acid and ethylmercaptan was evolved freely. The resulting

¹ Johns and Baumann: this *Journal*, xiv, p. 331, 1913.

² Kurt Sembritzki: *Ber. d. deutsch. chem. Gesellsch.*, xx, p. 1814, 1897.

³ Fischer and Ach: *ibid.*, xxviii, p. 2473, 1895.

⁴ E. Frankland Armstrong: *ibid.*, xxxiii, p. 2308, 1900.

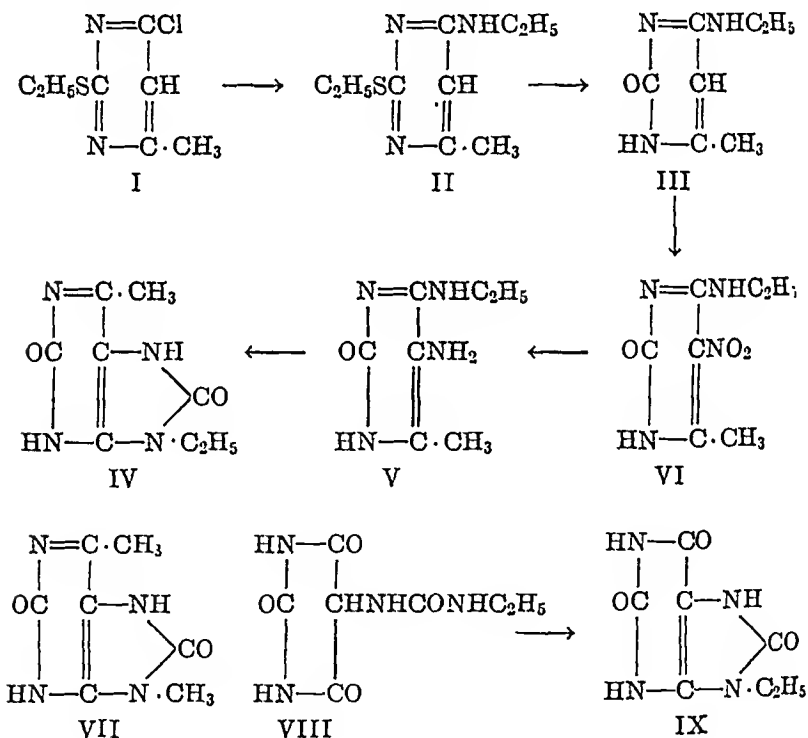
⁵ Johns: this *Journal*, ix, p. 161, 1911.

⁶ Johns: *Amer. Chem. Journ.*, xl, p. 351, 1908.

2-oxy-4-methyl-6-ethylaminopyrimidine (III) gave a nitro compound (VI) which could readily be reduced to 2-oxy-4-methyl-5-amino-6-ethylaminopyrimidine (V) by using freshly precipitated ferrous hydroxide as the reducing agent. A very good yield of 2,8-dioxy-6-methyl-9-ethylpurine (IV) was obtained by heating the 2-oxy-4-methyl-5-amino-6-ethylaminopyrimidine with urea.

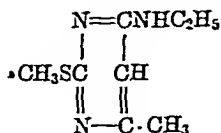
Since 2,8-dioxy-6-methyl-9-ethylpurine (IV) is a homologue of 2,8-dioxy-6,9-dimethylpurine (VII),⁷ we would expect the properties of these purines to be closely related, and we find this to be the case.

These researches will be continued.



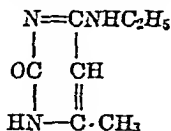
⁷Johns: this *Journal*, xi, p. 397, 1912.

EXPERIMENTAL PART.

2-Ethylmercapto-4-methyl-6-ethylaminopyrimidine.

Twenty grams of 2-ethylmercapto-4-methyl-6-chloropyrimidine⁸ were mixed with 28.8 grams of a 33 per cent aqueous solution of ethylamine and the mixture heated in a sealed tube at 90°-100°C. for three hours. The reaction product was obtained in the form of a heavy yellow oil. This was drawn off in a separatory funnel and washed with water. When the oil was scratched with a glassrod in a beaker it solidified to a crystalline mass. The yield was 20 grams or 95 per cent of the calculated amount, after being dried at room temperature. The substance was very soluble in alcohol, ether or benzene. It was also soluble in cold dilute acids but almost insoluble in dilute alkalis. It was slightly soluble in hot water, and on cooling the solution, it was precipitated as an oil. It was very soluble in boiling ligroin, and crystallized well in the form of colorless pointed prisms on cooling the solution slowly. The crystals thus obtained melted at 70°C.

	Calculated for $\text{C}_9\text{H}_{11}\text{N}_4\text{S}$	Found:
N.....	21.32	21.36

2-Oxy-4-methyl-6-ethylaminopyrimidine.

Twenty grams of 2-ethylmercapto-4-methyl-6-ethylaminopyrimidine were dissolved in 100 cc. of concentrated hydrochloric acid, and the solution was boiled gently under a reflux condenser for an hour. Ethylmercaptan was evolved rapidly. The solution was then evaporated to dryness on a steam bath. This treatment left

⁸ Johns: *Amer. Chem. Journ.*, xl, p. 351, 1908.

a hydrochloride of 2-oxy-4-methyl-6-ethylaminopyrimidine. This salt was dissolved in water and the solution was made slightly alkaline with ammonium hydroxide. On concentrating on the steam bath, clusters of crystals appeared on the surface of the liquid. The evaporation was then discontinued and the solution was cooled slowly. A bulky mass of crystals was obtained. The crystals were filtered off by suction and washed with a little cold water to remove ammonium chloride. The yield was 11.5 grams or 75 per cent of the calculated. A considerable amount remained in the mother liquor. The compound was very soluble in hot and moderately soluble in cold alcohol. It was insoluble in ether and slightly soluble in benzene. It was easily soluble in dilute acids and alkalies. It was very soluble in hot water and moderately soluble in cold water and from this solvent it crystallized in acicular prisms. These melted partially and decomposed at 245°–250°C.

	Calculated for $C_7H_{11}ON_3$:	Found:
N.....	27.45	27.25

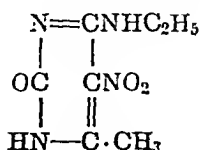
The hydrochloride of 2-oxy-4-methyl-6-ethylaminopyrimidine.



This compound was prepared by dissolving 1 gram of the base in 5 cc. of 20 per cent hydrochloric acid and concentrating until crystals could be obtained on cooling. When such a solution was cooled slowly, colorless diamond-shaped plates were obtained. These were filtered off and washed with concentrated hydrochloric acid and dried at about 80°C. They were very soluble in water. They melted to a clear oil at 214°–215°C. The yield was only about 0.2 gram from 1 gram of the base as the hydrochloride was soluble in dilute hydrochloric acid.

	Calculated for $C_7H_{11}ON_3 \cdot HCl$:	Found:
Cl.....	18.73	18.86

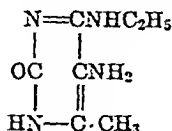
2-Oxy-4-methyl-5-nitro-6-ethylaminopyrimidine.



Five grams of 2-oxy-4-methyl-6-ethylaminopyrimidine were dissolved in 10 cc. of cold concentrated sulphuric acid. Heat was evolved as solution took place but the temperature was kept down to 60°-70°C. To the still warm solution were then added gradually 5 cc. of nitric acid, density 1.5. Heat was generated. When the addition of nitric acid was complete, the resulting solution was kept at 60°-70° for five minutes, and then poured into cold water. This solution was cooled and cautiously neutralized with concentrated ammonium hydroxide. As soon as an excess of ammonia was present, a yellow color appeared. This was removed by acidifying slightly with acetic acid. The white precipitate which formed, was filtered off by suction and washed with cold water and dried at 90°-100°C. The yield was 5.9 grams, which corresponds to 91 per cent of the calculated weight. The nitro compound dissolves in hot ammonium hydroxide and a yellow ammonium salt crystallizes out on cooling. It also dissolves in dilute alkalis forming yellow solutions. It is slightly soluble in hot alcohol or benzene and insoluble in ether. It dissolves sparingly in hot water and from this solvent it crystallizes in needles. It dissolves in dilute hydrochloric acid and in glacial acetic acid at room temperature. It begins to decompose slowly at about 238°C. and chars at 260°-265°C.

	Calculated for $C_7H_{11}O_2N_4$	Found:
N.....	28.28	28.14

2-Oxy-4-methyl-5-amino-6-ethylaminopyrimidine.



Five grams of 2-oxy-4-methyl-5-nitro-6-ethylaminopyrimidine were suspended in 100 cc. of water on a steam bath and 75 cc. of concentrated ammonium hydroxide were added. The nitro-pyrimidine dissolves on warming gently. A hot concentrated solution of 50 grams of crystallized ferrous sulphate was added. Reduction proceeded rapidly and was accompanied by the liberation of heat. The sulphate was precipitated by the addition of a concentrated solution of 60 grams of barium hydroxide and after thorough

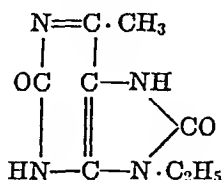
shaking the excess of baryta was precipitated by the addition of ammonium carbonate. The resulting mixture was kept warm for about one-half hour and then filtered. The precipitate was thoroughly washed with hot water. The filtrate and washings were then concentrated to a small volume, clarified once with blood coal and then concentrated to about 10 cc. About 3 grams of crystals were obtained on cooling the solution. More of the diamino-pyrimidine was obtained from the mother liquor, the total yield being 90–95 per cent of the calculated amount. The crystals were not soluble in ether and but slightly soluble in benzene. They were very soluble in hot alcohol or hot water and moderately soluble in cold water. They dissolved readily in cold alkalis or acids. An aqueous solution did not give a precipitate on adding barium chloride and did not form a difficultly soluble picrate. Mercuric chloride produced a white precipitate which became dark when the solution was boiled. An ammoniacal silver solution was reduced in the cold with the formation of a silver mirror. The substance crystallized from water in clusters of needles that contained one molecule of water of crystallization after drying over sulphuric acid. On drying for two hours at 130°C. the crystals became anhydrous.

1.8828 grams of substance lost 0.1803 gram of H_2O .

	Calculated for $C_7H_{13}ON_4 \cdot H_2O$	Found:
H_2O	9.67	9.57

	Calculated for $C_7H_{13}ON_4$	Found:
N.....	33.33	33.36 33.41

2,8-Dioxy-6-methyl-9-ethylpurine.



One gram of 2-oxy-4-methyl-5-amino-6-ethylaminopyrimidine and 1 gram of urea were pulverized together in a mortar, and the mixture was heated in an oil bath at 170°–180°C. for an hour. The

urea melted, leaving the pyrimidine partly suspended in the liquid, but on heating for about a half hour, the whole mass began to solidify. Very little charring occurred. The reaction product was dissolved in hot dilute ammonium hydroxide, producing a red solution. This was clarified with blood coal. The resulting solution was acidified with acetic acid whereupon the purine crystallized out. On cooling, filtering and washing out the salts with water, 0.9 gram of pure purine was obtained. This is 78 per cent of the calculated amount. The purine dissolved in about 35 parts of boiling water and in about 500 parts of water at room temperature. It was not soluble in ether and but slightly soluble in boiling benzene or alcohol. It dissolved readily in dilute alkalies and was moderately soluble in cold dilute acids. It crystallized from water in needles that formed sheaves like tyrosine. It did not form a difficultly soluble picrate and did not give a precipitate with mercuric chloride or barium chloride. When added to an ammoniacal silver solution it formed a jelly which did not seem to be changed by heating. When heated with concentrated nitric acid on the steam bath it oxidized and left a yellow film, but if sufficient purine was present, a blood-red residue was obtained. The yellow film turned red when it was moistened with ammonia and dried on the steam bath. The crystallized purine possessed a pearly lustre and was found to be anhydrous. It did not melt at 310°C .

0.2073 gram of substance gave 0.3790 gram of CO_2 and 0.0942 gram of H_2O .

	Calculated for $\text{C}_4\text{H}_4\text{O}_2\text{N}_4$:	Found:	
C	49.48	49.86	
H.....	5.04	5.08	
N.....	28.87	ⁱ 29.14	ⁱⁱ 29.08

THE INTERCONVERSION OF α -AMINO-ACIDS, α -HYDROXY-ACIDS AND α -KETONIC ALDEHYDES.

PART II.

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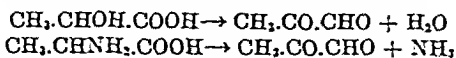
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1. Introduction.

The object of the following paper is to present the detailed experiments upon which we have based a hypothesis concerning the intermediary metabolism of amino- and hydroxy-acids, and in particular the mechanism concerning the mutual interconversion of alanine, lactic acid and glucose.¹ For the sake of clearness, we may reproduce the essential features of the types of reactions which we believe to be operative in the changes concerned.

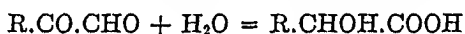
By making use of a substance capable of forming extremely insoluble derivatives with α -ketonic aldehydes, namely, paranitrophenylhydrazine, we have been able to show, by experiments *in vitro*, that amino-acids and hydroxy-acids, such as alanine and lactic acid, readily undergo decomposition in faintly acid solution in conformity with the following equations:



¹ This *Journal*, xiv, p. 555, 1913.

Amino-acids have been commonly regarded as extremely stable substances, at least *in vitro*, but our observations tend to show that under suitable conditions, in aqueous solution, when due provision is made for the prompt removal of the products of their decomposition, both the α -amino-acids and α -hydroxy-acids are in a state of unstable equilibrium.² Furthermore, we have been able to show that the decomposition of amino-acids with formation of ketonic aldehydes is not due to a complicated reaction dependent upon the presence of the nitrophenylhydrazine, for it has been possible to demonstrate ammonia formation from amino-acids under similar conditions, but in the absence of the hydrazine.

The production of α -ketonic aldehydes from α -amino- and α -hydroxy-acids is of biochemical significance, partly on account of the existence of enzymes which we have named "glyoxalases" capable of converting the former substances into hydroxy-acids.³



Moreover, we have been able to gather a considerable amount of indirect evidence indicating that α -ketonic aldehydes may play a part in intermediary metabolism. Thus we find that methyl glyoxal yields glucose in the glycosuric organism just as do alanine and lactic acid, from which methyl glyoxal may be derived *in vitro* (section 7).

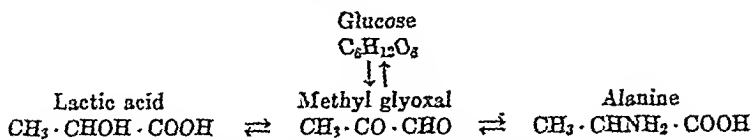
In addition, it is possible to demonstrate, *in vitro*, the reverse change, namely, the conversion of glucose into methyl glyoxal under conditions which, apart from temperature, are comparable with those existing in the animal body (section 4).

From the foregoing evidence and other that has been referred to in our previous paper, it appeared justifiable to construct a scheme which may crudely represent the interconversion of alanine, lactic acid, methyl glyoxal and glucose by a series of reversible reactions involving the addition or subtraction of water or ammonia. Of the various reactions indicated, the direct formation of alanine from methyl glyoxal is the only one that thus far has

² It is of interest to note that the β -amino-acids, such as β -alanine and β -phenylalanine, which do not occur in nature, do not yield ketonic aldehydes, at any rate under the above conditions.

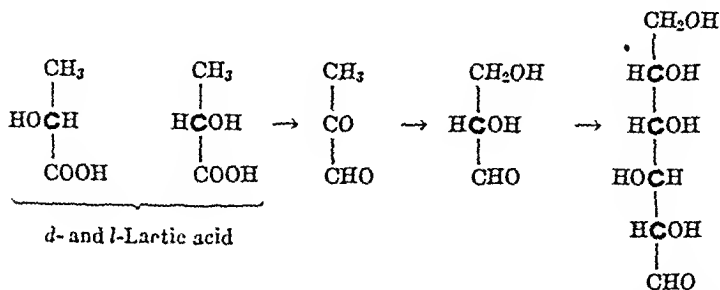
³ This *Journal*, xiv, p. 423, 1913.

not been demonstrated although the analogous synthesis of glycine from glyoxal has been effected.



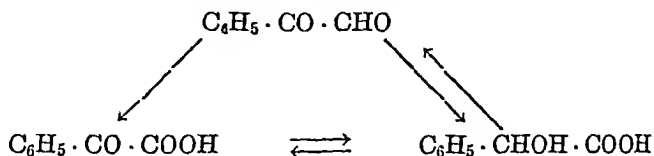
The lactic acid which we have obtained by the action of glyoxalase upon methyl glyoxal is a mixture of the two forms in which the laevo acid is in excess. The production of glucose from *d*-lactic acid and from the inactive acid is well established through Mandel and Lusk's experiments, but it appeared very desirable to determine whether the pure laevo acid might also yield glucose. Accordingly, we have prepared pure *l*-lactic acid from morphine *l*-lactate and find that it also yields glucose freely in the glycosuric animal.

This result appears to us to be of considerable significance, for the almost quantitative conversion of both *d*- and *l*-lactic acids, substances possessing asymmetric carbon atoms enantiomorphously related, into the same *d*-glucose apparently necessitates a loss of asymmetry in the lactic acid molecule in the process of glucose synthesis. The intermediate formation of methylglyoxal, such as we have suggested, would furnish a satisfactory explanation of such a change, and in addition, the conversion of methyl glyoxal into glucose, possibly with intermediate formation of glyceric aldehyde, would give an opportunity for the introduction of new asymmetric groups.



Finally, reference may be made to the relation of the α -ketonic acids to amino-acids and α -ketonic aldehydes. Neubauer and

Knoop have clearly demonstrated the interconversion of amino- and ketonic acids. A clue to the mechanism of this reaction may be furnished by our observations (section 6) on the formation of phenyl glyoxylic acid as well as *l*-mandelic acid on perfusing a liver with blood containing phenyl glyoxal.



It is possible that the phenyl glyoxylic acid originates as a secondary product of the oxidation of mandelic acid rather than by the direct oxidation of phenyl glyoxal.⁴ But in any case, the result is of interest since it serves to bring the α -ketonic aldehydes in close biochemical relation with the amino- as well as the hydroxy-acids.

In the experimental part of this paper it will be shown that the nitrophenylhydrazones of glyoxylic and pyruvic acids may be obtained by the direct action of nitrophenylhydrazine upon glycollic and lactic acids. Hydrazino-acids appear to be first formed and are readily oxidized to the ketonic acid derivatives in the presence of air. Although ordinary hydrazines are not known to occur in the animal body many substances such as arginine and creatine contain the $-\text{NH} \cdot \text{NH}_2$ group and it is conceivable that substances of this type may be concerned in the biochemical oxidation of hydroxy to ketonic acids.

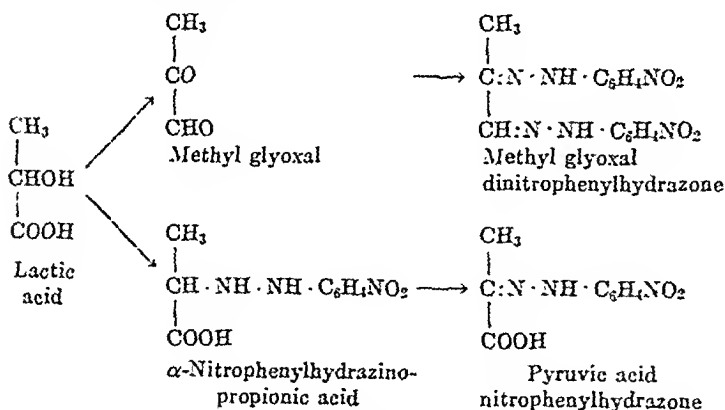
2. *The formation of methyl glyoxal from lactic acid.*

It is an extremely easy matter to demonstrate the formation of methyl glyoxal from lactic acid by simply allowing a filtered 5 or 10 per cent aqueous solution of lactic acid (500 cc.) containing a little nitrophenylhydrazine (1–2 grams) to stand at room temperature or in the incubator. After two or three hours, a flocculent red precipitate begins to appear, which in the earlier

⁴ It is of interest to note that Evans (*Amer. Chem. Journ.*, xxxv, p. 128, 1906) oxidized phenyl glyoxal to phenyl glyoxylic acid by means of alkaline permanganate. In the absence of alkali benzoic acid is obtained.

stages of the reaction, is composed of almost pure methyl glyoxal dinitrophenylhydrazone.

The precipitate gradually increases in amount during the succeeding three or four days, when it will be found that all the nitrophenylhydrazine has disappeared, although the amount of precipitate does not account for nearly all of the base added.⁵ When this stage has been reached, it is well to filter off the precipitate on a small funnel and to dissolve an additional quantity of nitrophenylhydrazine (1-2 grams) in the filtrate by warming and subsequently cooling and filtering from any trace of insoluble matter. The clear filtrate, on standing, soon begins to deposit more methyl glyoxal dinitrophenylhydrazone and the process may be repeated as often as desired. After a time it will be noticed, however, that the character of the precipitate begins to change and that a yellowish-brown crystalline substance begins to deposit in addition to the amorphous methyl glyoxal derivative. The crystalline deposit is a mixture of nitrophenylhydrazinopropionic acid and the nitrophenylhydrazone of pyruvic acid, the latter being formed from the former by oxidation. The changes may be represented as follows:



The separation of the constituents of the red precipitate may be conveniently carried out as follows:

The mixture is first of all washed with hot 10 per cent sodium

⁵ Unchanged nitrophenylhydrazine is conveniently tested for by adding a drop of acetone or benzaldehyde to a few cubic centimeters of the solution.

carbonate solution which dissolves the nitrophenylhydrazino-propionic acid and the pyruvic acid nitrophenylhydrazone. The residue is then washed with warm alcohol and finally dissolved in a small amount of boiling nitrobenzene, filtered hot and toluene added to the filtrate. The pure methyl glyoxal dinitrophenylhydrazone separates out almost immediately in the form of glistening crimson needles which are filtered off, washed with toluene and dried at 140° . The substance was identical in every way with the hydrazone prepared directly from methyl glyoxal and melted with decomposition at about 302° – 304° after darkening slightly above 290° . On warming the merest trace of the substance with caustic soda solution, best with the addition of a few drops of alcohol, there develops a magnificent deep blue color slowly changing to purple, then violet and finally a dull brown-red. The reaction is extremely sensitive.

ANALYSIS: 0.1218 gram dried at 150° gave 0.0299 gram N = 24.6 per cent N.
 $C_{15}H_{14}O_4N_6$ requires 24.6 per cent N.

For purposes of comparison, the nitrophenylhydrazone of methyl glyoxal was prepared directly from methyl glyoxal obtained by the hydrolysis of its acetal (Meisenheimer). A slight excess of the hydrazine (2.2 mols.) dissolved in 30 per cent acetic acid was added to methyl glyoxal (1 mol.). The hydrazone is at once precipitated in practically theoretical amount and may be washed with alcohol and then recrystallized as above from nitrobenzene and toluene. The substance is very sparingly soluble in almost all solvents with the exception of nitrobenzene and bases such as pyridine. It should be noted that nitrobenzene and especially pyridine, combine with the substance very tenaciously and are only given off *in vacuo* at 100° with extreme slowness. Heating at 140° – 150° is much more efficient in driving off the solvents. Pyridine seems to be a somewhat more objectionable solvent than nitrobenzene on account of the ease with which it unites with the hydrazone forming dark red-brown solutions. The pure substance melts at 302° – 304° , the exact temperature varying slightly with the speed of heating. On analysis the substance was found to contain 25.1 per cent nitrogen (theory = 24.6). The same substance has been described by Neuberg,⁶ who obtained it from α -aminopropionic

⁶ *Ber. d. deutsch. chem. Gesellsch.*, xli, p. 956, 1908.

aldehyde. The melting point is given as 277° with discoloration at 255° . We believe this melting point to be much too low. The reaction with caustic soda was not described.

The α -nitrophenylhydrazinopropionic acid and pyruvic acid nitrophenylhydrazone contained in the sodium carbonate washings from the original precipitate were recovered on acidifying with acetic acid. On repeated recrystallization from boiling water, the first substance is slowly oxidized to the pyruvic acid derivative, so that probably the former was not obtained perfectly pure. It is fairly soluble in alcohol, melts above 250° and gives an intense red coloration with caustic soda.

ANALYSIS: 0.0919 gram substance gave 0.0169 gram N = 18.3 per cent N.
 $C_9H_{11}O_4N_2$ requires 18.6 per cent N.

By repeated crystallization from water of the mixture of the hydrazino acid and the pyruvic acid nitrophenylhydrazone, several grams of the latter were readily obtained as a bright yellow crystalline substance melting at 223° – 225° . It is also formed by oxidizing nitrophenylhydrazinopropionic acid with an ammoniacal solution of a cupric salt. It is moderately soluble in alcohol and gives a bright red color on addition of caustic soda.

ANALYSIS: 0.1225 gram gave 0.0223 gram N = 18.6 per cent N.
 $C_9H_9O_4N_2$ requires 18.8 per cent N.

The substance obtained from lactic acid was identical with the product obtained from pyruvic acid as described by Hyde⁷ and also prepared for comparison by us. The melting point given by Hyde is 219° – 220° .

A number of experiments were made in which additions of other substances were made to the lactic acid mixture in the hope of accelerating its decomposition into methyl glyoxal and water. The following were tried: spongy platinum, aluminum oxide, chromium oxide, mercuric iodide, uranium acetate, glycine, sulphuric acid and calcium lactate. None of them proved effective.

It should be noted that while the yield of crystalline substances from the lactic acid is small, the greater part of the acid remains unchanged and may be treated over and over again with fresh nitrophenylhydrazine.

⁷ *Berichte*, xxxii, p. 1815.

3. *The formation of methyl glyoxal and ammonia from alanine.*

The formation of methyl glyoxal from alanine is readily demonstrated by allowing an aqueous solution of the amino-acid with a little nitrophenylhydrazine and a few drops of an acid to stand at room temperature, or better in the incubator at 39°. After a few hours, the separation of a red precipitate commences and its quantity gradually increases from day to day. From time to time it is advisable to filter off the precipitate and to add more nitrophenylhydrazine. If no acid be added to the mixture, a precipitate is still obtained but it contains little or none of the methyl glyoxal derivative. Comparative experiments in which equivalent amounts of sulphuric and acetic acids were used, failed to show any marked difference.

In one experiment, a filtered solution containing alanine (25 grams), nitrophenylhydrazine (1.5 grams) and acetic acid (5 cc.) and water (500 cc.) was digested at 39°. After three hours, the separation of a precipitate was noticeable and the amount gradually increased during the following four days, when almost all the nitrophenylhydrazine had disappeared. The precipitate was filtered off and additional nitrophenylhydrazine (1 gram) dissolved in the filtrate. A second precipitation soon commenced and the whole process was eventually repeated four times.

The combined precipitates which weighed rather less than a gram, were purified by washing successively with hot 10 per cent sodium carbonate solution, water and alcohol. The residue was then crystallized from a mixture of nitrobenzene and toluene and was obtained in the form of red needles melting at 302° identical with methyl glyoxal dinitrophenylhydrazone prepared from other sources. It gave the color reaction with caustic soda in typical fashion. On mixing the substance from alanine with a preparation from methyl glyoxal, the melting point of the mixture was unchanged.

ANALYSIS: 0.1367 gram gave 0.0333 gram N = 24.4 per cent N.
 $C_{15}H_{14}O_4N_2$ requires 24.6 per cent N.

In addition to the above typical experiment, we have made a number of others in which the reaction and concentration of the acid was varied but without obtaining materially different results.

The yield of the methyl glyoxal derivative is small but it must be remembered that most of the alanine may be recovered unchanged, so that it is likely that the yield is relatively large compared with the amount of amino-acid decomposed.

The formation of methyl glyoxal from alanine necessitates the simultaneous liberation of ammonia and we have made a number of experiments which indicate that small amounts of ammonia are liberated from amino-acids with much greater ease than has been commonly supposed. We find, for example, that if a weak solution (2 per cent) of ordinary sodium phosphate is boiled in a distilling flask attached to a condenser until the distillate is perfectly free from ammonia when tested with Nessler's reagent and then a gram or so of an amino-acid, such as alanine, is added, the second distillate will be found to contain very definite traces of ammonia. On continuing the distillation, the amount of ammonia slowly diminishes but does not disappear entirely, and on allowing the previously boiled mixture to stand in the distilling flask for a short time (*e.g.*, 1 hour), a fresh formation of ammonia is apparent. Similar results were obtained on substituting sodium borate, prepared from boiled ammonia-free caustic soda and ignited boric acid, for the phosphate.

On adding a little freshly distilled ammonia-free acetic acid to a dilute alanine solution which has been well boiled with a little caustic soda to remove any ammonia present as an impurity and then digesting the mixture in the distillation apparatus at about 50° for an hour or two, we find that on making alkaline with caustic soda and redistilling, there is no difficulty in detecting ammonia in the distillate. Digestion of amino-acids with weak caustic soda solution ($\frac{N}{100}$) also appears to lead to ammonia formation.

It need hardly be added that in all of the above experiments appropriate blank tests were constantly carried out and every effort made to guard against accidental contamination. We propose to study the reaction quantitatively.

4. *The formation of methyl glyoxal from glucose.*

Methyl glyoxal was shown by Pechmann to be somewhat volatile with steam and we made use of this property for its isolation from the complex mixture of substances resulting from the action of salts upon glucose.

Glucose (50 grams) and sodium phosphate crystals (25 grams) were dissolved in water (500 cc.) and the mixture was distilled until about 300 cc. of distillate were obtained. An addition of 300 cc. of 5 per cent phosphate was then made and the distillation repeated until finally about 3 liters of distillate were obtained. The distillate gave a marked iodoform reaction and on treatment with *p*-nitrophenylhydrazine dissolved in acetic acid gave a red flocculent precipitate. The precipitate was collected and crystallized from either pyridine or better from nitrobenzene in deep crimson needles melting at 300°. The melting point was unchanged on mixing with pure methyl glyoxal dinitrophenylhydrazone.

ANALYSIS: 0.0882 gram dried at 150° gave 0.0214 gram N = 24.3 per cent N.
 $C_{15}H_{14}O_4N_6$ requires 24.6 per cent N.

The yield of precipitate was small, about 0.5 gram, but no doubt only a very small proportion of the glyoxal formed was obtained in the distillate. Reference may be made here to the interesting experiments of Henderson⁸ upon the loss of optical activity of glucose solutions on digestion with phosphates. The reaction undoubtedly deserves careful study. It is possible that the methyl glyoxal derivative we obtained is derived from acetol, but while this is doubtful it is not a matter of great importance for the purpose of the present experiments.

5. *The formation of other α -ketonic aldehydes from α -hydroxy-acids and α -amino-acids.*

We have been able to observe the formation, from a number of hydroxy- and amino-acids, of insoluble nitrophenylhydrazones giving reactions indicative of their being derived from α -ketonic aldehydes; but in many cases we must defer an accurate description of the properties of the substances until we have had opportunity to study them more closely. The experiments were conducted in similar fashion to those already described with lactic acid and alanine, so that repetition will be unnecessary.

Glycollic acid. Glycollic acid (20 grams), *p*-nitrophenylhydrazine (2 grams) and water (200 cc.) were heated together, cooled, filtered and then digested at 39°. After a day a fine red granular

⁸ This *Journal*, x, p. 3, 1911.

precipitate separated out which gave all the reactions of glyoxal dinitrophenylhydrazone. The quantity of precipitate increased steadily, but after a few days bright yellow crystals began to separate, which dissolved in caustic soda to give a bright red color. They proved to be the nitrophenylhydrazone of glyoxylic acid. The precipitate which first separated crystallized from nitrobenzene and toluene in small glistening deep red crystals melting with evolution of gas at 302° . On mixing the substance with glyoxal dinitrophenylhydrazone prepared from glyoxal, the melting point was unchanged. A trace of the substance on warming with caustic soda solution and a few drops of alcohol, gives a transitory greenish-blue color, passing to a deep blue and slowly changing to violet and finally brown red. The yield of pure substance was insufficient for analysis.

For comparison, glyoxal dinitrophenylhydrazone was prepared from glyoxal precisely as in the case of the methyl glyoxal derivative. The yield is practically theoretical. The substance has also been obtained by Wohl and Neuberg from glycollic aldehyde.⁹

The precipitate appearing during the later stages of the glycollic acid digestion was filtered off and washed with water and then dissolved in much boiling alcohol. On filtering, a small red precipitate consisting chiefly of glyoxal dinitrophenylhydrazone separated out. On concentrating the filtrate, yellow crystals of the nitrophenylhydrazone of glyoxylic acid were obtained. This substance has an indefinite melting point, beginning to decompose at a temperature somewhat above 200° , and on repeated crystallization, passes over into a less soluble modification, which is very sparingly soluble even in boiling nitrobenzene. The hydrazone dissolved in caustic soda to give a bright red color and was identical with the substance previously described by one of us, which was prepared directly from glyoxylic acid.¹⁰

Glyceric acid. The conversion of glyceric acid in 5 or 10 per cent solution on digestion with nitrophenylhydrazine (1 per cent) into the nitrophenylosazone of glyceric aldehyde is effected with remarkable ease. The osazone separates out in abundance after one to two hours and its quantity steadily increases as long as unchanged nitrophenylhydrazine is present.

⁹ *Ber. d. deutsch. chem. Gesellsch.*, xxxiii, p. 3107, 1900.

¹⁰ *This Journal*, iv, p. 235, 1903.

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The precipitate was filtered off and recrystallized from nitrobenzene and toluene. The osazone crystallizes in long thin scarlet needles melting at about 315° with evolution of much gas. It is sparingly soluble in alcohol, ether or amyl alcohol.

ANALYSIS: 0.1292 gram gave 0.0301 gram N = 23.3 per cent N.
 $C_{15}H_{14}O_6N_6$ requires 23.8 per cent N.

A trace of the substance boiled with caustic soda and a little alcohol gives successively greenish-blue, deep blue, violet red and brown-red colors.

An attempt to prepare the above osazone from glyceric aldehyde, for purposes of comparison, gave a disappointing yield.

Mandelic acid. Experiments with mandelic acid similar to those with glycollic acid, gave a complicated mixture of substances. On washing the precipitate successively with hot sodium carbonate solution, water and alcohol, and then recrystallizing the residue from nitrobenzene, a small quantity of substance was obtained which resembled phenyl glyoxal dinitrophenylhydrazone in every respect. The amount of pure substance was insufficient for analysis.

The sodium carbonate washings on acidification gave a small quantity of phenyl glyoxylic acid nitrophenylhydrazone, crystallizing in hair-like needles melting at 163° – 165° . Both of the above-mentioned hydrazones were prepared by independent methods for purposes of comparison.

Phenyl glyoxal dinitrophenylhydrazone was prepared from phenyl glyoxal (1 mol.) and nitrophenylhydrazine (2.2 mols.) dissolved in acetic acid (33 per cent). The red precipitate was washed with alcohol and recrystallized from nitrobenzene and toluene. It crystallizes in bright red needles melting at 302° – 304° .

ANALYSIS: 0.1551 gram gave 0.3370 gram CO_2 and 0.0559 gram H_2O .

	Found:	Calculated for $C_{20}H_{14}N_4O_6$
C.....	59.3	59.4
H.....	4.1	4.0

A trace of the substance warmed with caustic soda and alcohol gives successively carmine red, purple, clear light red fading finally to a light brown color.

Phenyl glyoxylic acid nitrophenylhydrazone was prepared by adding nitrophenylhydrazine dissolved in a slight excess of 5 per cent sulphuric acid to phenyl glyoxylic acid.¹¹ The hydrazone is precipitated as a yellow substance which crystallizes from weak alcohol, in which it is readily soluble, in hair-like needles melting at 163°–165°. It dissolves in caustic soda to give a bright red color.

ANALYSIS: 0.1056 gram substance gave 0.01568 gram N = 14.8 per cent N.
 $C_{14}H_{11}N_2O_4$ requires 14.7 per cent N.

Glycine. The experiments with this substance were similar in every respect to those with alanine. There was no difficulty in detecting the formation of glyoxal dinitrophenylhydrazone, but the yield of precipitate was distinctly smaller than in the case of alanine.

Aspartic acid. On digesting a 1 per cent solution of aspartic acid with nitrophenylhydrazine at 39°, there is an abundant formation of a dinitrophenylhydrazone. The substance crystallizes from nitrobenzene and toluene in the form of small thick prisms, and on warming with caustic soda gives successively a greenish-blue, clear deep blue, followed by a more persistent violet-blue, finally turning reddish brown. The substance is apparently the dinitrophenylhydrazone of the α -ketonic aldehyde corresponding to aspartic acid ($COOH.CH_2.CO.CHO$). It will be studied further.

We have also obtained dinitrophenylhydrazones from other amino-acids including valine, leucine, phenylalanine, proline. These substances are sparingly soluble compounds with high melting points and give characteristic color reactions with caustic soda. We prefer to reserve their detailed description until we have had an opportunity of studying them more closely.

¹¹ A most convenient method for the preparation of phenylglyoxylic acid is as follows: Mandelic acid (10 grams) is neutralized with caustic potash and diluted to 500 cc. with ice and water. Potassium permanganate in 4 per cent solution (200 cc.) is added drop by drop to the cooled potassium mandelic solution, using a mechanical stirrer. Half an hour after all the permanganate has been added sulphur dioxide is passed in to dissolve the oxides of manganese. Sulphuric acid is then added in excess and the phenylglyoxylic acid extracted with ether. The yield is 90 per cent of the calculated one. (Cf. Evans: *loc. cit.*)

6. *The fate of methyl glyoxal and phenyl glyoxal on perfusion through the liver. The formation of phenyl glyoxylic acid.*

The perfusions of dogs' livers with methyl and phenyl glyoxals were carried out as in the case of similar experiments reported from this laboratory,¹² with the exception that sodium phosphate was added to the perfusion mixture in order to provide for the prompt neutralization of any acid that might be formed.

Methyl glyoxal. The dog (14 kgm.) was starved for two days before operation. The liver was perfused for half an hour with a mixture containing blood, 500 cc.; phosphate, 500 cc. 5 per cent; methyl glyoxal, 3 grams; salt solution, 250 cc. After perfusion, the mixture was heated to coagulate protein and the liver was also cut up and boiled with water and the filtrates combined. The filtrates were evaporated almost to dryness, acidified with phosphoric acid and then taken up with gypsum. The dry powder was extracted with ether in the usual way. The ether extract was taken up in water and was found to be strongly laevo-rotatory. It was boiled with zinc carbonate and gave two crops of dextro-rotatory zinc lactate (4 grams). The rotations and analyses showed that both *d*- and *l*-lactic acids were present, the latter being in excess.

Crop I. (2.1 grams): 0.3302 gram dried at 120° lost 0.0585 gram H₂O = 17.7 per cent.

ROTATION: 0.2667 gram air dried salt in 10 cc.; *l* = 2 dm.; $\alpha = 0.1^\circ$.
 $[\alpha]_D = + 2.28^\circ$

Crop II. (1.9 grams): 0.2025 gram dried at 120° lost 0.0340 gram H₂O = 16.8 per cent.

ROTATION: 0.1685 gram of dry salt in 10 cc.; *l* = 2 dm.; $\alpha = 0.20^\circ$.
 $[\alpha]_D = + 5.93^\circ$

0.2702 gram of the mixed salts gave 0.0904 gram ZnO = 33.5 per cent ZnO.
 C₆H₅O₆ requires 33.4 per cent ZnO.

A second perfusion was made in which no methyl glyoxal was added. The dog (7 kgm.) had not been starved and the liver contained much glycogen. 500 cc. of 5 per cent phosphate solution were added to the blood, which after a perfusion lasting half an hour was analyzed as before. 1.7 grams of zinc lactate were obtained, all of which was derived from dextro lactic acid.

¹² This *Journal*, ix, p. 146, 1911.

ANALYSIS: 0.2516 gram dried at 120° lost 0.0335 gram H_2O = 13.2 per cent.

- 0.2150 gram gave 0.0714 gram ZnO = 33.2 per cent.

ROTATION: 0.2838 gram in 10 cc.; l = 2 dm.; α = -0.42° .

$[\alpha]_D = -8.53^\circ$.

Phenyl glyoxal. Two experiments were made with phenyl glyoxal which were essentially similar to the methyl glyoxal experiment. In one experiment, 3 grams of phenyl glyoxal and 200 cc. of 5 per cent phosphate were added to the blood saline mixture, and perfusion carried on for one and three quarters hours. In the second experiment 4 grams of phenyl glyoxal and 400 cc. of phosphate were added and perfusion lasted one hour.

The aqueous filtrates from blood and liver were concentrated and, after acidifying with phosphoric acid, extracted with ether in a continuous extractor. The ethereal solution in each case was shaken twice with 10 cc. of saturated sodium bisulphite solution to separate any phenyl glyoxylic acid. The main ether extract was then evaporated to dryness and the crystalline residue of mandelic acid recrystallized from boiling toluene. The yield of mandelic acid varied from 1-1.6 grams. It was practically all the laevo-rotatory variety and melted at 131° .

ROTATION: 0.5776 gram in 20 cc.; l = 2 dm.; α = -8.52° .

$[\alpha]_D = -148^\circ$.

The sodium bisulphite extracts were strongly acidified with sulphuric acid and extracted with ether. The ether residue, in addition to phenyl glyoxylic acid, contained much mandelic acid which apparently may be extracted from ether solutions by sodium bisulphite to a rather surprising extent.

The residue gave the benzene, thiophene, sulphuric acid test for phenyl glyoxylic acid in typical fashion.¹³ The acid was further characterized as the nitrophenylhydrazone. The residue was dissolved in water, filtered and a clear solution of nitrophenylhydrazine in dilute sulphuric acid added. A bright yellow precipitate of phenyl glyoxylic acid *p*-nitrophenylhydrazone at once separated and was purified by recrystallization from dilute alcohol. In one experiment 0.2 gram was obtained, in the second 0.1 gram. The

¹³ Some samples of technical benzene do not contain enough thiophene to give the reaction. It is therefore advisable to add thiophene separately.

substance melted at 163°–165° and was identical with the product prepared directly from phenyl glyoxylic acid (section 5).

ANALYSIS: 0.1100 gram gave 0.0161 gram = 14.6 per cent N.
 $C_{11}H_{11}N_3O_4$ requires 14.7 per cent N.

It may be noted here that on simple digestion of muscle tissue extracts with phenyl glyoxal, we have been able to detect readily the formation of phenyl glyoxylic acid.

7. The fate of methyl glyoxal and of l-lactic acid in the glycosuric organism.

For these experiments, we made use of dogs rendered glycosuric by daily injections of phlorhizin (1 gram) suspended in olive oil. The conditions of the experiments were similar to those of recently published experiments.¹⁴

Methyl glyoxal. A preliminary experiment was made in which 1.5 grams of methyl glyoxal in aqueous solution were given subcutaneously to a rabbit (1.5 kgm.) without effect, showing that it was relatively non-toxic. The methyl glyoxal used for the following experiment was freshly prepared by hydrolyzing the acetal according to Meisenheimer's method. It was given by stomach tube and produced no particular symptoms. The nitrophenylhydrazine test showed that no unchanged methyl glyoxal was excreted in the urine. The urine was collected in six-hour periods.

NITROGEN	GLUCOSE	G : N	ACETOACETIC ACID	SUBSTANCE GIVEN
3.67	14.40	3.92	0.012	9 gms. methyl glyoxal
3.16	19.37	6.13	0.018	
4.43	17.18	3.88	0.066	
		3.81		

The rise in G:N ratio on giving the methyl glyoxal is very marked. Using 3.87 as the average ratio, it is calculated that 9 grams of methyl glyoxal gave a little over 7 grams of "extra glucose."

In a second experiment in which the methyl glyoxal was given by subcutaneous injection, the G:N ratio rose from 3.7 to 7.66.

¹⁴ This *Journal*, xiv, p. 321, 1913.

l-Lactic acid. The acid was prepared by resolving inactive lactic acid with morphine according to Irvine's¹⁵ excellent method. The crystalline morphine *l*-lactate was decomposed by ammonia and the alkaloid filtered off. The ammonium lactate was converted into the calcium salt by prolonged boiling with lime. The pure crystallized calcium *l*-lactate was finally decomposed by heating with an equivalent weight of sodium sulphate and the calcium sulphate removed by filtration. The sodium lactate was given by stomach tube and evoked no symptoms.

NITROGEN	GLUCOSE	G:N	ACETOACETIC ACID	SUBSTANCE ADDED
3.74	14.21	3.81	0.155	12 gms. <i>l</i> -lactic acid as sodium salt.
3.25	20.68	3.80	0.043	
		6.33		
3.72	13.10	3.52	0.013	
		3.55		

Adopting 3.62 as the average G:N ratio, it is found that 12 grams of lactic acid furnished slightly over 9 grams of glucose.

¹⁵ *Transactions of the Chem. Soc.*, lxxxix, p. 935, 1906.

THE CHEMISTRY OF GLUCONEOGENESIS.

V. THE RÔLE OF PYRUVIC ACID IN THE INTERMEDIARY METABOLISM OF ALANINE.

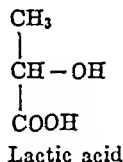
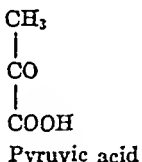
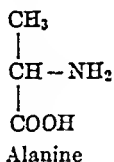
BY A. I. RINGER.

WITH THE ASSISTANCE OF E. M. FRANKEL AND L. JONAS.

(From the Department of Physiological Chemistry of the University of Pennsylvania, Philadelphia, Pa.)¹

(Received for publication, May 27, 1913.)

Pyruvic acid occupies a singularly important position in intermediary metabolism. Because of its chemical relationship to alanine and lactic acid



it is assumed to play a rôle in the intermediary metabolism of both protein and carbohydrates.

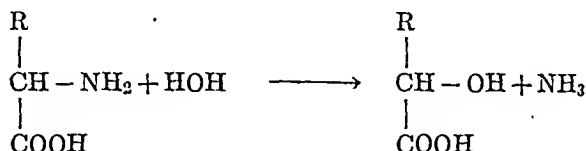
Not until recent years was any attempt made to study the paths that the amino-acids undergo in the process of their catabolism. All that was known was that the amino-acid broke down into a nitrogenous fraction that gave rise to urea and ammonia, and a "nitrogen-free" fraction which was "burnt."

With the development of our knowledge of the structural composition of the amino-acids and their related compounds, evidence began to accumulate which suggested definite reactions and definite paths of decomposition. Until the researches of Neubauer² came to light, it was the current belief that the α -amino-acids suffered deamination in the animal body by a process of hydrol-

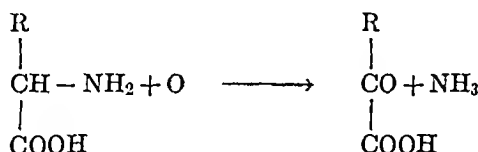
¹ Aided by a grant from the Rockefeller Institute for Medical Research.

² O. Neubauer: *Deutsch. Arch. f. klin. Med.*, xcv, p. 211, 1909.

ysis, whereby the NH_2 was removed and an hydroxyl took its place.



Neubauer was the first to call attention to a different process of deaminization, *i.e.*, oxidative deaminization,

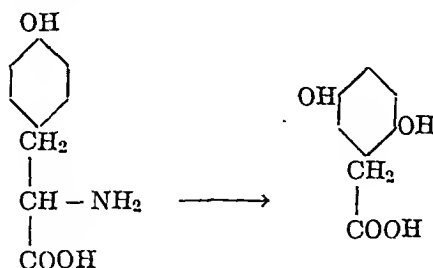


which has so much experimental evidence to support it that it is now almost universally accepted.

We shall not attempt to present a detailed account of the experiments which led to this conclusion, but will briefly state the facts that have a bearing on our present discourse.

Neubauer worked on a patient suffering from alkaptonuria. Such a patient presents an abnormality in his protein metabolism, which consists of his inability to burn tyrosine and phenylalanine and of the excretion of homogentisic acid in the urine. Neubauer and his associates utilized this fact in their study of the intermediary metabolism of tyrosine and phenylalanine³ in the course of which they came to the following conclusions:

I. That tyrosine (para-oxyphenyl- α -amino-propionic acid) gives rise to extra homogentisic acid⁴



³ O. Neubauer and W. Falta: *Zeitschr. f. physiol. Chem.*, xlii, p. 81, 1904.

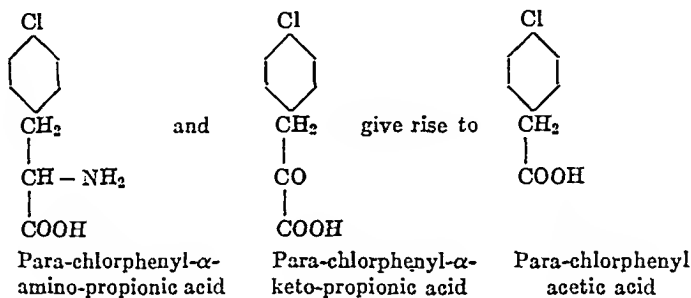
⁴ Wolkow and Baumann: *ibid.*, xvi, p. 270, 1892.

II. That para-oxyphenyl- α -hydroxy-propionic acid, $\text{HO.C}_6\text{H}_4\text{.CH}_2\text{.CHOH.COOH}$, does not give rise to extra homogentisic acid.

III. That para-oxyphenyl- α -keto-propionic acid, $\text{HO.C}_6\text{H}_4\text{CH}_2\text{.CO.COOH}$, gives rise to extra homogentisic acid.

Since these α -amino and α -keto compounds can give rise to homogentisic acid, and the α -hydroxy cannot, Neubauer concluded that the α -amino compound cannot possibly give rise to the α -hydroxy acid as an intermediary body. He then conceived of the "oxidative deamination" theory, which seems to explain his findings beautifully.

Additional support to this theory was rendered by Friedmann and Masse⁵ who showed that



whereas para-chlorophenyl- α -hydroxy-propionic acid, $\text{Cl.C}_6\text{H}_4\text{.CH}_2\text{.CHOH.COOH}$, did not give rise to para-chlorophenyl acetic acid. This again showed that the hydroxy acid could not possibly have been an intermediary compound in the metabolism of the amino-acid.

On the strength of his findings Neubauer was led to extend his theory to the entire series of α -amino-acids and suggested that alanine, in the animal body, gives rise to pyruvic acid, which may secondarily give rise to lactic acid.

Reviewing the evidence in support of this theory, one feels convinced of the soundness of the conclusion in the case of the aromatic compounds. With regard to the open chain α -amino-acids, however, there seems to be little direct evidence. It is true that the conversion of α -keto-acids into alanine by the ani-

⁵ Friedmann and Masse: *Biochem. Zeitschr.*, xxvii, p. 97, 1910.

mal organism has been proven and that ketonic acids may in the animal body go over with great ease into the corresponding hydroxy acids, which process has been shown repeatedly to be reversible; still we feel that *it is not proven satisfactorily that alanine in its catabolism must pass through pyruvic acid, and that lactic acid can arise only secondarily from pyruvic acid.*

We raise this question because in experiments which we have performed with the object of ascertaining the degree to which pyruvic acid can give rise to extra glucose in diabetic animals, it was found that pyruvic acid does not behave in the way it would if it really occupied a definite and obligatory place in the intermediary metabolism between alanine and lactic acid.

During the course of our work we have come across several substances whose gluconeogenetic properties are not constant, which have the power of yielding more glucose at one time and less at other times, and we have been inclined to attribute their gluconeogenetic properties to certain factors of equilibrium, which turn the reaction of their metabolism in one path at one time and in another path at another time. According to our experience pyruvic acid may be classed among this group.

The status of pyruvic acid in metabolism has been the subject of very considerable discussion during the past year. Parnas and Baer⁶ perfused the liver of the tortoise with Ringer's solution, blood and oxygen to which 4 grams of pyruvic acid as sodium salt had been added, and found no increase in the glycogen. On the basis of this one experiment they drew the conclusion that pyruvic acid cannot be classed among the glucogenetic substances. In our estimation, this conclusion is not at all justified.

P. Mayer⁷ administered 7 to 8 grams of pyruvic acid to normal rabbits and found that they developed glucosuria, the severity of which depended upon the state of nutrition of the animal. In one rabbit he obtained 2.4 grams of glucose in the twenty-four hours following the administration of pyruvic acid. In animals which had fasted for periods of ten to eleven days, the administration of pyruvic acid was not followed by glucosuria, but by an increase of the glucose concentration of the blood and by an increase in the glycogen in the liver. In this communication Mayer left open the question of the origin of the glucose. In a second paper

⁶ Parnas and Baer: *Biochem. Zeitschr.*, xli, p. 386, 1912.

⁷ Paul Mayer: *ibid.*, xl, p. 441, 1912.

on the subject⁸ (which appeared after our work was far advanced) Mayer studied the influence of pyruvic acid on gluconeogenesis in phlorhizinized dogs and rabbits. He found that pyruvic acid did not give rise to any extra glucose, and that in some cases the urinary constituents were greatly diminished after the pyruvic acid administration. In experiment X the glucose elimination dropped from 13.48 grams in the fore period to 1.45 grams (!) in the experimental period, and the nitrogen dropped from 2.92 grams to 0.53 gram (!). In experiment XI the glucose elimination dropped from 32.93 grams to 3.41 grams (!). The kidneys of these animals were examined microscopically and extensive tubular degeneration was found. Mayer concluded that pyruvic acid is a toxic substance which causes a disturbance in the sugar and nitrogen elimination and which acts by decreasing the permeability of the kidneys.

To all of these conclusions we object most emphatically. We gave Kahlbaum's pyruvic acid seven times to six different dogs in quantities varying from 8.8 to 13.2 grams, administered subcutaneously and orally, and never have we obtained any of the toxic symptoms described by Mayer. In no case did we get the peculiar drop in the glucose and nitrogen eliminations, and in no case did we observe any sign or symptom of any kidney involvement. We have, however, seen a picture of Mayer's experiences with pyruvic acid after subcutaneous and, under certain circumstances also, after oral administration of tartaric acid.⁹ The resemblance is so close that we do not hesitate for a moment to attribute Mayer's results to a contamination of his pyruvic acid with tartaric acid. This is all the more probable since pyruvic acid is very largely prepared by the distillation of tartaric acid.

In all of our experiments pyruvic acid appears to be a glucose-yielding substance, the question is only one of degree. The methods employed in these experiments are the same as those employed and described in the previous papers of this series.

In experiment XXII period VIII, 10 grams of pyruvic acid as sodium salt were administered subcutaneously. The glucose elimination rose from 33.6 grams in the fore period to 40.91 grams in the experimental period, and returned to 35.75 and 32.54 grams in

⁸ Paul Mayer: *ibid.*, xlix, p. 486, 1913.

⁹ Underhill: this *Journal*, xii, p. 115, 1912. We have been able to corroborate Underhill's findings. Our results will be published soon.

the after periods IX and X respectively. The amount of extra glucose eliminated was 8.21 grams.

In experiment XXIII period III, 10 grams of pyruvic acid as sodium salt were given subcutaneously. The glucose elimination rose from 28.54 to 32.04 grams and returned to 22.84 grams in the after period. The D:N ratio, which was 3.44 in the fore period, rose to 4.18 and came down to 3.6 in the after period. The amount of extra glucose was 5.09 grams.

In experiment XXIV period III, 13.2 grams of pyruvic acid as sodium salt were given subcutaneously. The glucose elimination rose from 17.58 grams to 19.18 grams. The amount of extra glucose eliminated was 2.25 grams.

In experiment XXV period IV, 8.8 grams of pyruvic acid as sodium salt were given subcutaneously. The amount of extra glucose eliminated was 5.1 grams.

In experiment XXVI period V, 8.8 grams of pyruvic acid as sodium salt were given subcutaneously. The amount of extra glucose eliminated was 1.16 grams.

In two other experiments, 10 grams of pyruvic acid as sodium salt were given *per os*. The dog vomited part of the ingested material each time. The animals lived for a long time afterwards and no change in the urinary constituents was noticed. The experiments are not reported in detail because the vomitus contaminated part of the urine.

From all these experiments we see very clearly that pyruvic acid cannot be considered a toxic substance (in Mayer's sense) and that in most of the cases pyruvic acid yields large quantities of extra glucose.

However, when we come to compare the results obtained after pyruvic acid administration with those obtained after alanine¹⁰ and lactic acid,¹¹ we note a very marked difference. While alanine and lactic acid never fail to yield large quantities of extra glucose, pyruvic acid at times yields very small quantities (experiments XXIV and XXVI). This fact makes it very certain that *in the process of catabolism alanine cannot have pyruvic acid as its principal product of intermediary metabolism, and that alanine does not undergo oxidative deaminization*. Alanine and lactic acid yield glucose in quantities so similar to each other, that one seems

¹⁰ Ringer and Lusk: *Zeitschr. f. physiol. Chem.*, lxvi, p. 106, 1910.

¹¹ Mandel and Lusk: *Amer. Journ. of Physiol.*, xvi, p. 129, 1906.

justified in concluding that the conversion of the former into the latter is quantitative.

In a previous communication¹² it was shown that malic acid yields glucose in quantities similar to aspartic acid, and it was suggested that the former was an intermediary product in the metabolism of the latter. It becomes of interest to know what rôle, if any, the corresponding ketone—oxalacetic acid—plays in it. These experiments are in progress and will be reported soon.

EXPERIMENT XXII. *Twelve-hour periods.*

DATE 1913	PERIOD	WEIGHT	TOTAL NITROGEN	TOTAL GLUCOSE	D:N	EXTRA GLUCOSE	NIHN	ACETONE AND ACETO-ACETIC ACID	REMARKS
Feb.									
2	IV		9.82	38.38	3.91		0.89	1.25	
3	V		9.12	33.60	3.68		0.88	1.04	
3	VI	17.40	8.04						
4	VII		8.00						
4	VIII	17.00	8.65	40.91	4.73	8.21	0.505	0.308	10 gms. pyruvic acid as Na salt given subcutaneously.
5	IX		9.18	35.75	3.89		0.50	0.279	
5	X	16.64	8.62	32.54	3.77		0.49	0.283	

EXPERIMENT XXIII. *Twelve-hour periods.*

3	II	12.40	8.28	28.54	3.44			0.340	
3	III		7.66	32.04	4.18	5.09		0.160	10 gms. pyruvic acid as above.
4	IV		6.34	22.84	3.60			0.430	
4	V	11.54	6.13	23.38	3.81			0.501	
5	VI		5.81	20.51	3.52			0.840	
5	VII	11.20	5.32	20.08	3.77			0.514	
6	VIII		5.21	18.40	3.53				

¹² Ringer, Frankel and Jonas: this *Journal*, xiv, p. 539, 1913.

EXPERIMENT XXIV. *Twelve-hour periods.*

DATE 1913	PERIOD	WEIGHT	TOTAL NITROGEN	TOTAL GLUCOSE	D : N	EXTRA GLUCOSE	NH ₃ N	ACETONE AND ACETO-ACETIC ACID	β -OXY BUTYRIC ACID	REMARKS
Apr. 30	I		4.77	18.45	3.87		0.27	0.07	0.24	13.2 gms. pyruvic acid as above
May 1	II		5.58	17.58	3.16		0.36	0.11	0.47	
1	III	8.00	5.26	19.18	3.65	2.25	0.36	0.13	0.39	
2	IV		5.74	18.75	3.27		0.29	0.10	0.40	
2	V	7.98	4.47	15.00	3.36		0.19	0.13	0.49	

EXPERIMENT XXV. *Twelve-hour periods.*

May 1	I	9.31	3.77	12.63	3.35			0.22	1.21	8.8 gms. pyruvic acid as above.
2	II		3.85	12.10	3.14		0.41	0.39	2.14	
2	III	9.11								
3	IV		3.34	13.80	4.14	5.10	0.23	0.18	0.69	
3	V	8.94	3.30	11.60	3.52		0.23	0.35	1.82	
4	VI		4.40	13.10	2.98		0.35	0.60	2.56	

EXPERIMENT XXVI. *Twelve-hour periods.*

May 16	IV		7.80	28.60	3.68			0.13	0.35	8.8 gms. pyruvic acid as above.
16	V	12.44	8.35	32.30	3.87	1.16		0.09	0.34	
17	VI		7.82	29.51	3.78			0.22	1.00	

SUMMARY.

I. Experiments on phlorhizinized dogs have shown that pyruvic acid is capable of yielding extra glucose in the diabetic organism.

II. In some cases the quantity of glucose was found to be much less than arises from similar amounts of alanine and lactic acid.

III. It is concluded that pyruvic acid cannot be considered a necessary intermediary product in the conversion of alanine into lactic acid and that alanine cannot be considered to undergo oxidative deamination.

SPHINGOMYELIN.

FIRST PAPER.

ON THE PRESENCE OF LIGNOCERIC ACID AMONG THE PRODUCTS OF HYDROLYSIS OF SPHINGOMYELIN.

By P. A. LEVENE.

(From the Rockefeller Institute for Medical Research, New York.)

(Received for publication, May 28, 1913.)

The products of hydrolysis of sphingomyelin have not been the subject of a careful investigation in recent years. The knowledge of the acid components of the substance is limited to the statement of Thudichum¹ that the principal acid taking part in the formation of the molecule is a saturated fatty acid, of the composition $C_{13}H_{26}O_2$, melting at $59^{\circ}C.$, and hence isomeric with stearic acid. In course of the present investigation it was found that the acid had the composition $C_{21}H_{42}O_2$, a melting point of $81^{\circ}C.$, and formed an ethyl ester melting at $55-56^{\circ}C.$, and hence was lignoceric acid. A sample of the ester mixed with that of an ester of lignoceric acid showed the same melting point. The molecular weight of the acid was 368.

EXPERIMENTAL PART.

Sphingomyelin in lots of 40 grams was taken up in 400 cc. of alcohol containing 7 per cent of sulphuric acid and heated with reflux condenser on the water bath for six hours. The product of hydrolysis was allowed to remain at room temperature ($20^{\circ}C.$) over night, and the ester separated in form of beautiful scales. These were filtered, again taken up in alcohol containing 7 per cent of sulphuric acid, and heated as before for an additional five hours. The ester which separated on the second esterification was filtered off with suction, and recrystallized out of acetone at room

¹ A Treatise on the Chemical Constitution of the Brain; London, 1884.

temperature. Filtered and dried in a vacuum desiccator it had a sharp melting point of 55–56°C. Mixed with a sample of an ester of lignoceric acid obtained on oxidation of cerebronic acid it showed the same melting point of 55–56°C.

0.1428 gram of the substance gave on combustion after Dennstedt 0.4110 gram of CO_2 and 0.1686 gram of H_2O .

	Calculated for $\text{C}_{21}\text{H}_{41}\text{COOC}_2\text{H}_5$:	Found:
C.....	78.80	78.50
H.....	13.10	13.20

For estimation of the molecular weight the ester was saponified by means of alcohol containing an $\frac{N}{2}$ solution of sodium hydrate.

1.3491 grams of the substance neutralized on boiling for four hours 3.57 cc. of $\frac{N}{2}$ alkali. M. W. = 395.

1.3734 grams of the substance neutralized under the same conditions 3.50 cc. of $\frac{N}{2}$ alkali. M. W. = 391.

1.7848 grams of the substance neutralized 4.55 cc. of alkali. M. W. = 391.

	Calculated for $\text{C}_{21}\text{H}_{41}\text{O}_2$:	Found:		
		I	II	III
M. W.....	368	367	368	363

In order to obtain the free acid the ester was saponified by means of alcoholic sodium hydrate. The soap was decomposed by means of hydrochloric acid, and the free acid repeatedly taken up in water containing hydrochloric acid and warmed on the water bath until the acid melted to an oil. The free acid was finally transformed into the lead salt, and the lead salt decomposed by means of sulphurated hydrogen. The free acid was recrystallized out of toluene. It melted sharply at 81°C., and had the following composition.

0.1160 gram of the substance gave on combustion after Dennstedt 0.3331 gram of CO_2 and 0.1366 gram of H_2O .

	Calculated for $\text{C}_{21}\text{H}_{41}\text{O}_2$:	Found:
C.....	78.30	78.33
H.....	13.00	13.03

ON CHONDROITIN SULPHURIC ACID.

SECOND PAPER.

BY P. A. LEVENE AND F. B. LA FORGE.

(From the Rockefeller Institute for Medical Research, New York.)

(Received for publication, May 29, 1913.)

It was stated in the previous communication¹ on this subject that chondrosin is composed of two substances, both in some way related to carbohydrates. One was identified as *d*-glucuronic acid; regarding the nature of the other component the views of different writers were most contradictory. Thus, Schmiedeberg² was led to the belief that the substance was glucosamine and Orgler and Neuberg³ announced it to be monoamino-tetrahydroxycaproic acid. Indeed these authors have described crystalline salts of the acid, which were supposedly obtained from the products of barium hydrate hydrolysis of the complex. The most recent reference to the nature of the substance was made by Fränkel,⁴ who regarded it as an amino-glucuronic acid.

Some of the properties of the chondrosin seemed to justify the view of Orgler and Neuberg. Namely, the substance displayed a marked resistance towards boiling mineral acids; and, on the other hand, was readily hydrolyzed by alkalies. For this reason an attempt was made to repeat the experiment of Orgler and Neuberg. Soon, however, it became evident that success could not be expected from these experiments for the reason that even at the low temperature of the thermostat room (40°C.) an aqueous solution of barium hydrate caused chondrosin to give up all its nitrogen in form of ammonia. At this stage of the investigation it was concluded to search for some indirect evidence re-

¹ This *Journal*, xv, p. 69.

² Schmiedeberg: *Arch. f. exp. Path. u. Pharm.*, xxviii, p. 358, 1891.

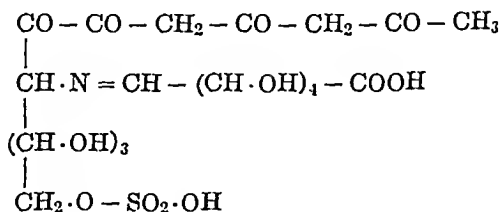
³ Orgler and Neuberg: *Zeitschr. f. physiol. Chem.*, xxxvii, p. 407, 1903.

⁴ S. Fränkel: *Ann. d. Chem.*, cccli, p. 344, 1907.

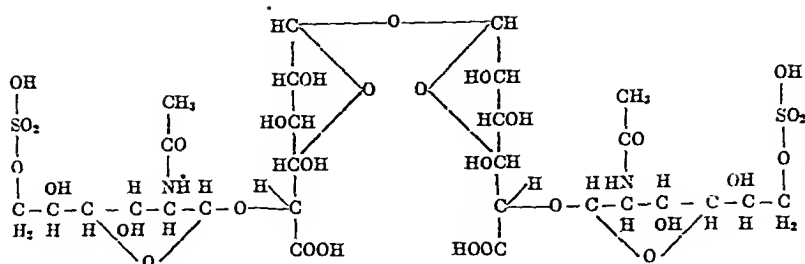
garding the nature of the substance, and for this reason an attempt was made to obtain laevulinic acid on treatment of chondroitin sulphuric acid with strong mineral acids. It was shown by Neuberg that glucosamine under certain conditions yields laevulinic acid particularly after preceding deaminization with nitrous acid. Chondrosin was therefore deaminized by means of sodium nitrite and hydrochloric acid, and the product boiled with mineral acids. Two experiments were performed and each yielded small quantities of laevulinic acid. This observation encouraged a renewal of the efforts to obtain glucosamine on direct treatment of chondroitin sulphuric acid with mineral acid. Similar to other workers we met with failures when the experiments were performed on the crude acid sodium salt of chondroitin sulphuric acid. The experiment reported in this communication was performed on a barium salt of chondroitin sulphuric acid free from any mineral impurities. The result of the experiment was most gratifying, as glucosamine hydrochloride was obtained, without an effort, in analytically pure condition.

Thus, for the first time all the components of chondroitin sulphuric acid were isolated and identified. They were those assumed on indirect evidence by Schmiedeberg, namely, sulphuric acid, acetic acid, glucosamine and glucuronic acid.

However, the results of the present investigation necessitate a revision of Schmiedeberg's view regarding the chemical structure of the complex molecule. According to Schmiedeberg's view the structure of the substance is presented by the following graphic formula:



The results of the present investigation lead to the following graphic expression of its composition:



The two views differ in the following. The theory of Schmiedeberg assumes the union of the glucosamine and of glucuronic acid through the formation of a nitrogen bridge. This view is erroneous for the reason that in chondrosin the nitrogen is present in the form of a free amino group, and hence can be removed by nitrous acid. On the basis of the present work one may assume a glucosidic union between the glucosamine and one of the secondary alcohol groups of the glucuronic acid. Of these the γ -position is excluded by virtue of the γ -oxidic structure of glucuronic acid.

The second difference of the two theories relates to the number and place of the acetyl groups entering in the structure of the molecule. Schmiedeberg assumed the presence of three acetyl groups in chondroitin, basing his view exclusively on the percentage composition of the amorphous salts of the substance.

In the course of the present work it was not possible to detect more than one acetyl group on hydrolysis of chondroitin sulphuric acid with mineral acids, or with an aqueous solution of barium hydrate. The presence of only one acetyl group was suggestive on the basis of analytical data, showing in chondroitin sulphuric acid a ratio of $N:C = 1:14$. The same ratio was found by Levene and Jacobs⁵ on the analysis of the barium salt of glycothionic acid obtained from tendo-mucin.

The place of the acetyl group is made obvious by the fact that the nitrogen of chondroitin sulphuric acid is not removed in form of nitrogen gas by nitrous acid, and it was stated already that

⁵ Levene and Jacobs: *Journ. of Exp. Med.*, x, p. 557, 1908.

chondrosin contains its nitrogen in form of an unsubstituted amino group.

Finally, the theory expressed here assumes a glucosidic union of two chondrosin molecules, for the reason that this view offers the simplest explanation to the observation that chondroitin does not possess reducing power for Fehling's solution, while chondrosin does.

EXPERIMENTAL.

Laevulinic acid from chondrosin.

Fifty grams of chondroitin sulphuric acid barium salt were converted into chondrosin. The solution deaminized with sodium nitrite and evaporated to about 75 cc. in vacuum. Two volumes of 40 per cent hydrochloric acid were then added and the solution boiled for twenty hours. It was then filtered from melanin and extracted several times with ether. The ethereal extract was dried with sodium sulphate, evaporated to dryness and the residue taken up in 250 cc. of water. It contained tin which was removed with hydrogen sulphide and the filtrate, after having been evaporated to remove the latter, was again extracted with ether. The residue from the ethereal solution was taken up in about 2 cc. of water nearly neutralized with ammonia, and the chlorine was removed with a few drops of silver nitrate solution. The addition of concentrated silver nitrate and ammonia to the filtrate produced a white precipitate which was washed with alcohol and ether and dried. The yield was 0.2 gram.

0.1260 gram substance gave 0.0602 gram Ag.

	Calculated for $C_6H_7O_4Ag$:	Found:
Ag	48.24	48.57

Glucosamine hydrochloride from chondrosin.

Fifty grams of chondroitin sulphuric acid barium salt were converted into chondrosin, the solution evaporated to a syrup in vacuum and taken up into 150 cc. of water. 150 cc. of concentrated hydrochloric acid were then added together with 10 grams of stannous chloride, and the solution boiled for six hours under a reflux condenser. Without filtering from the separated melanin, the product was diluted to 500 cc. and the tin removed with hydro-

gen sulphide. The colorless filtrate from the tin sulphides was concentrated in vacuum to a thick syrup which crystallized spontaneously to a solid cake. This was ground with 95 per cent alcohol in a mortar, filtered and washed repeatedly with alcohol and finally with ether and dried in vacuum. The yield was 9 grams; 2 grams crystallized from the alcoholic mother liquors upon standing.

0.1481 gram of substance gave 17.7 cc. amino nitrogen at 20°, 766 mm.

0.1226 gram substance from alcoholic mother liquors washed and dried in vacuum gave 0.1512 gram CO₂ and 0.0750 gram H₂O.

0.1608 gram of substance recrystallized from 80 per cent alcohol with addition of a few drops of concentrated HCl required 7.72 cc. AgNO₃ solution (1 cc. = 0.00352 Cl).

	Calculated for C ₄ H ₁₂ O ₅ N HCl:	Found:
N	6.51	6.87
Cl	16.45	16.90
C	33.40	33.63
H	6.54	6.79

0.1001 gram substance in 10 cc. H₂O rotated in a 1 dm. tube with D-light + 1°.

$$[\alpha]_{20}^D = 99.9.$$

Nitrogen-carbon ratio in chondroitin sulphuric acid barium salt.

0.5346 gram of barium salt dried to constant weight in vacuum at 100° gave 8.25 cc. of $\frac{N}{10}$ NH₃.

0.2197 gram of barium salt gave 0.0880 gram H₂O and 0.2110 gram CO₂.

N	2.18 per cent.
H	4.45 per cent.
C	26.18 per cent.

$$N:C = 1:14.1$$

Acetic acid determinations.

Twenty-five grams of the barium salt of chondroitin sulphuric acid were re-purified by dissolving in 2 liters of distilled water with the addition of 10 grams of pure barium chloride and precipitating by the addition of 1 liter of 95 per cent alcohol. The precipitate was washed chlorine-free with 50 per cent alcohol and finally with absolute alcohol and ether, and dried in vacuum. About 10 grams of this material were dissolved in 10 cc. of water.

The acetic acid determinations were carried out in the following manner. The solution of the chondroitin sulphuric acid barium salt was subjected to simultaneous hydrolysis with 25 per cent sulphuric acid and distillation at ordinary pressure. The distillate was collected in a receiver, cooled with ice and protected from the atmospheric carbon dioxide by soda lime, while the volume of the solution in the reaction flask was kept constant by the addition of water through a dropping funnel.

5 cc. solution gave 7.40 cc. $\frac{N}{10}$ NH_3 (Kjeldahl).

5 cc. solution gave 7.60 cc. $\frac{N}{10}$ acetic acid.

5 cc. solution gave 7.80 cc. $\frac{N}{10}$ acetic acid.

5 cc. solution gave 7.55 cc. $\frac{N}{10}$ acetic acid.

Five cubic centimeters of another solution, which corresponded to 8.50 cc. $\frac{N}{10}$ NH_3 (Kjeldahl) were hydrolyzed in the same apparatus with 20 per cent barium hydrate until no more ammonia was given off. The solution was then acidified with sulphuric acid and the acetic acid determination carried out as above.

5 cc. solution gave 9.75 cc. $\frac{N}{10}$ acetic acid.

STUDIES ON COTTON SEED MEAL TOXICITY. II.

IRON AS AN ANTIDOTE.

By W. A. WITHERS AND J. F. BREWSTER.

WITH THE COLLABORATION OF R. S. CURTIS, G. A. ROBERTS, L. F.
WILLIAMS AND J. W. NOWELL.

(From the North Carolina Agricultural Experiment Station, Raleigh.)

(Received for publication, May 31, 1913.)

It is a well established fact that cotton seed meal will produce death when fed for long periods and in large amounts to swine and smaller animals, and that care must be exercised even when feeding it too freely to cattle. This experiment station has been investigating this subject for some time and has made publication¹ of some of the previous work.

In the course of our experiments we were led to the hypothesis that the toxic principle² of cotton seed meal was a constituent group of the protein molecule containing loosely bound sulphur, and that the toxic effect of the meal was due to the action of this group upon the iron of the blood.

We reserve for a subsequent paper our experiments already performed and in process as they bear upon this hypothesis, but we desire in this paper to present the results of our experiments as they bear upon the efficiency of iron as an antidote for cotton seed meal poisoning.

In order to ascertain the toxicity of cotton seed meal under the conditions existing in our laboratory we fed meal to several rabbits until each died. Molasses was fed with the meal to increase the palatability. We began this feeding on April 23, 1911.

¹ *Science*, xxxvi, pp. 31-32, 1912; *Proceedings of the Society for the Promotion of Agricultural Science*, 1912, pp. 19-21; this *Journal*, xiv, pp. 53-58, 1913; Report of the North Carolina Agricultural Experiment Station, 1911-1912, pp. 141-149.

² This was announced in a paper read before the North Carolina Academy of Science, April 26, 1913, at its meeting in Greensboro, N. C.

Fifteen grams of cotton seed meal were given as the daily feed for each rabbit, which was approximately 10 grams per kilogram. The amount refused was weighed and deducted from the whole. Meal was fed at night and green feed in the morning. The feeding was carried on in galvanized iron cages. All in all we have fed cotton seed meal to twenty-two rabbits the average initial weight of which was a little over 1.5 kgms. As a rule the rabbits ate the meal well during the first few days and made gains in weight. But towards the end they began to refuse the meal in whole or in part and soon thereafter died. One rabbit died as early as six days after beginning to eat the meal, one survived for twenty-two days, but none beyond that time. On an average death ensued on the thirteenth day. Every rabbit lost in weight, the average loss for the period being about one-fifth of the initial weight of the animal. The average consumption of meal for each rabbit was 10 grams daily, or a total of about one-twelfth of the average initial weight of the rabbits.

The record of each rabbit is shown in the following table.³

Table showing toxicity of cotton seed meal to rabbits.

NUMBER OF RABBIT	WEIGHT OF RABBIT			COTTON SEED MEAL CON-SUMED	NO. OF DAYS SURVIVED FEEDING
	Initial	Final	Loss		
500	975	850	125	105	8
501	1225	1085	140	183	13
502	1130	1030	100	132	13
503	1320	1077	243	225	16
504	970	795	175	84	8
505	1370	1120	250	108	9
523	1580	1050	530	219	16
524	2370	1600	770	150	14
525	1670	1200	470	162	21
534	2560	1768	792	218	16
535	1940	1360	580	137	13
538	1605	1230	375	158	12
598	1154	1100	54	75	7
600	1307	1122	185	77	11
601	1591	1085	506	48	14
602	1260	1212	48	60	6
603	1260	912	348	71	16

³ All weights in this paper refer to grams.

Table showing toxicity of cotton seed meal to rabbits—Continued.

NUMBER OF RABBIT	WEIGHT OF RABBIT			COTTON SEED MEAL CONSUMED	NO. OF DAYS SURVIVED FEEDING
	Initial	Final	Loss		
681	2440	2082	358	209	22
745	2000	1200	800	92	14
746	1700	1350	350	131	13
850	1640	1430	210	143	13
851	1630	1570	60	132	11
Average.	1577.1	1237.6	339.5	132.7	13

Iron as an antidote for toxicity of cotton seed meal.

During our experiments we were feeding to some rabbits the residue left after treating cotton seed meal in the cold with an aqueous solution of sodium carbonate (3.8 grams dissolved in 286 cc. of water to 90 grams of meal, the treatment being for twenty-four hours). Each rabbit was getting the equivalent of 15 grams of whole meal. On the fourteenth day of the feeding two of the rabbits were dead, one was sick and all had lost in weight. From this we inferred that the feed was toxic. To test the effect of iron as an antidote to the poisonous effect of the feed, we began on the fourteenth day to supply in addition to this feed a solution of citrate of iron and ammonia (0.7 gram in 10 cc. of water at first each day and later on alternate days). This addition of iron to the feed was continued for fourteen days. The sick rabbit consumed the iron at once and appeared to be well the next day. From the time he was given iron he ate all the meal supplied to him except on the first day, when he refused part of it. The rabbits which had not been sick never refused any meal during the period after they began to receive iron. All three of them gained in weight during the fourteen-day period in which iron was given with the meal fraction whereas during the previous fourteen-day period in which the same fraction was fed without iron they had lost in weight. During the iron-feeding period each rabbit remained well, but during the previous feeding period without iron two had died and one was made sick. The contrast between the two feeding periods and the detailed records for each rabbit are shown in the following table.

Table showing the effect of feeding a cotton seed meal residue without iron.

RABBIT NUMBER	WEIGHT OF RABBIT			COTTON SEED MEAL CONSUMED	DAYS FED	CONDITION OF RABBIT AT END OF PERIOD
	Initial	14th day	Loss			
844	1430	1220	210	195	14	Died 14th day.
845	1800	1600	200	150	14	
846	1750	1620	130	165	14	Sick 14th day.
847	1760	1680	80	192	14	
848	1520	1400	120	150	11	Died 11th day.
Average	1652	1504	148	170		

Table showing the results of feeding iron with the cotton seed meal fraction for fourteen days.

RABBIT NUMBER	WEIGHT OF RABBIT			COTTON SEED MEAL CONSUMED	IRON SALT CONSUMED	DAYS FED	CONDITION OF RABBIT AT END OF PERIOD
	14th day	28th day	Gain				
845	1600	1710	110	210	7.7	14	Alive and normal.
846	1620	1670	50	203	7.7	14	Alive and normal.
847	1680	1700	20	210	7.7	14	Alive and normal.
Average.	1633	1693	60	208	7.7	14	

This feed was discontinued fourteen days after beginning the administration of iron. At that time the rabbits were apparently perfectly healthy.

This experiment indicates that iron may overcome the toxic effects of cotton seed meal where they have appeared, and if fed beforehand may prevent their appearance.

Effect of feeding iron with whole cotton seed meal.

As early as we could secure them we began feeding 15 grams of cotton seed meal daily to each of six rabbits, and along with the daily feed we gave 0.3 gram of citrate of iron and ammonia. For four days previous to the meal feeding we began to supply the iron solution. The detail is shown in the following table.

RABBIT NUMBER	WEIGHT OF RABBITS			FEED CONSUMED		DAYS FED	CONDITION OF RABBIT AT END OF PERIOD
	Initial	23d day	Gain	Cotton seed meal	Iron salt		
870	1370	1655	285	345	8.1	23	Alive and normal.
871	1550	1700	150	345	8.1	23	Alive and normal.
872	1420	1625	205	345	8.1	23	Alive and normal.
873	1330	1550	220	345	8.1	23	Alive and normal.
874	1110	1405	295	345	8.1	23	Alive and normal.
875	1500	1630	130	345	8.1	23	Alive and normal.
Average.	1380	1594	214	345	8.1	23	

It will be seen that each rabbit ate all the cotton seed meal and all the iron salt supplied and each gained in weight. By referring to the table at the beginning of this paper it will be seen that each of these six rabbits ate more cotton seed meal when iron was fed with the meal than any of the twenty-two rabbits could eat without the addition of iron. It will be noticed also by referring to the same table that every one of the twenty-two rabbits was killed by cotton seed meal before the twenty-third day if iron was not added, whereas all the six animals receiving iron along with the cotton seed meal are alive on the twenty-third day, are gaining in weight and are normal so far as we can judge.⁴ This experiment would indicate the great efficiency of iron as an antidote for cotton seed meal poisoning.

Confirming these results with six rabbits we have results obtained by feeding to a pair of rabbits a solution of citrate of iron and ammonia with whole cotton seed meal.

RABBIT NUMBER	WEIGHT OF RABBIT			FEED CONSUMED		DAYS FED	CONDITION OF RABBIT AT END OF PERIOD
	Initial	40th day	Gain	Cotton seed meal	Iron salt		
852	1720	1890	170	600	13.65	40	Alive and normal.
853	1580	1780	200	600	13.65	40	Alive and normal.
Average.	1650	1835	185	600	13.65	40	

⁴Each of these rabbits was alive and normal after forty-one days' feeding, each having consumed 615 grams of cotton seed meal and 13.5 grams of iron salt.

Comparing the results obtained with this feed and those obtained with the whole meal feed without iron it will be noted: that the iron-fed rabbits consumed nearly five times the amount of meal which proved fatal to the average rabbit without iron, and more than twice as much as the greatest amount of meal necessary to kill the rabbit which ate the most meal; that the iron-fed rabbits have survived more than three times as long as the average rabbit without iron, and nearly twice as long as the hardiest rabbit; that every rabbit fed iron and meal gained in weight and each rabbit fed meal without iron lost in weight.

It will be noted that each one of the twenty-two rabbits receiving cotton seed meal without iron died, that each of the eleven rabbits receiving iron along with cotton seed meal lived and gained in weight.⁵

These experiments lead us to suggest iron salts as an antidote to the toxicity of cotton seed meal.

Experiments are in progress with swine and results will be published later.

⁵ Each of these rabbits was alive and normal after fifty-eight days' feeding, each having consumed 870 grams of cotton seed meal and 19.95 grams of iron salt.

THE NECESSITY OF CERTAIN LIPINS IN THE DIET DURING GROWTH.

BY E. V. MCCOLLUM AND MARGUERITE DAVIS.

(From the Laboratory of Agricultural Chemistry of the University of Wisconsin.)

(Received for publication, June 1, 1913.)

Whether or not the growing mammal must obtain certain lipins preformed in the diet, has up to the present time not been definitely determined. That bodies of this class can be dispensed with for a considerable period without interfering with normal growth, is now well established. Osborne and Mendel¹ have recently published data showing that young rats can grow normally during sixty days on rations containing but insignificant traces of ether-soluble matter.

That lipins of several kinds can be synthesized in large quantities in the animal body, is apparent from the experiments of McCollum, Halpin and Drescher,² who secured normal egg production in hens on a ration containing but very small amounts of ether-soluble substances. This observation has been fully confirmed by Fingerling³ with ducks.

During the past year we have been engaged in a study of the influence of the composition and quantity of the inorganic content of the ration on growth in the rat.⁴ In this work we have employed rations compounded of pure casein, carbohydrates, and salt mixtures made up of pure reagents, and the same rations in which a part of the carbohydrates was replaced by lard, with a considerable degree of success. Young rats have been found to be very sensitive to variations in the character of the salt mix-

¹ This *Journal*, xii, p. 81, 1912.

² McCollum, Halpin and Drescher: Proceedings of the American Society of Biological Chemists, this *Journal*, xi, p. xiii, 1912; also *ibid.*, xii, p. 219, 1912.

³ Fingerling: *Biochem. Zeitschr.*, xxxviii, p. 448, 1912.

⁴ See McCollum and Davis: Proceedings of the American Society of Biological Chemists, this *Journal*, xiv, p. xl, 1913.

tures supplied, but with certain mixtures we have been able to obtain practically normal growth for periods varying from 70 to 120 days. Beyond that time little or no increase in body weight can be induced with such rations. These rats may remain in an apparently good nutritive condition on these rations for many weeks after growth ceases. That they are still capable of growth has been repeatedly demonstrated by changing to naturally occurring food-stuffs. That our animals, during their period of growth or during the period of suspension of growth which always accompanies long continued feeding of purified food substances, are in a physiological state which is nearly normal is evident from the fact that we have had three female rats produce young after being fed only casein, carbohydrates, lard and salt mixtures, for periods of 108, 127 and 142 days, respectively. These rats had made approximately normal growth for about eighty days on this ration. In none of these cases did the mothers produce enough milk to properly nourish the young, so that they were found to be decidedly undersized when seven to eighteen days old.

The fact that a rat of 40 to 50 grams in weight can grow normally during three months or more on such rations, then cease to grow but maintain its weight and a well nourished appearance for weeks and then resume growth on a ration containing certain naturally occurring food-stuffs would lead one to the belief that on these mixtures of purified food substances the animals run out of some organic complex which is indispensable for further growth but without which maintenance in a fairly good nutritive state is possible.

After numerous attempts to prevent the occurrence of growth suspension by nice adjustments between the various ingredients of our diets, we have found that the failure of rats to make further growth, after being brought to this "critical" point on mixtures of isolated food substances, is due to a lack of certain ether-soluble substances in the diet. These can be supplied by the ether extract of egg or of butter. The curves of a number of rats are presented to illustrate how a resumption of growth at about the normal rate results from the introduction of such ether-soluble substances into the diet after growth has ceased. It should be borne in mind that these experiments were intended primarily to furnish data as to the values of the salt mixtures

supplied by the rations and do not in all cases represent the highest degree of success attained by feeding these rations. The curves are selected entirely with a view to illustrating our almost invariable success in inducing a resumption of growth after complete suspension for a time on a ration which during eight weeks or more had sufficed for growth somewhat closely approximating the normal rate. The ether extracts employed were freed from ether *in vacuo* at temperatures below 60°C.

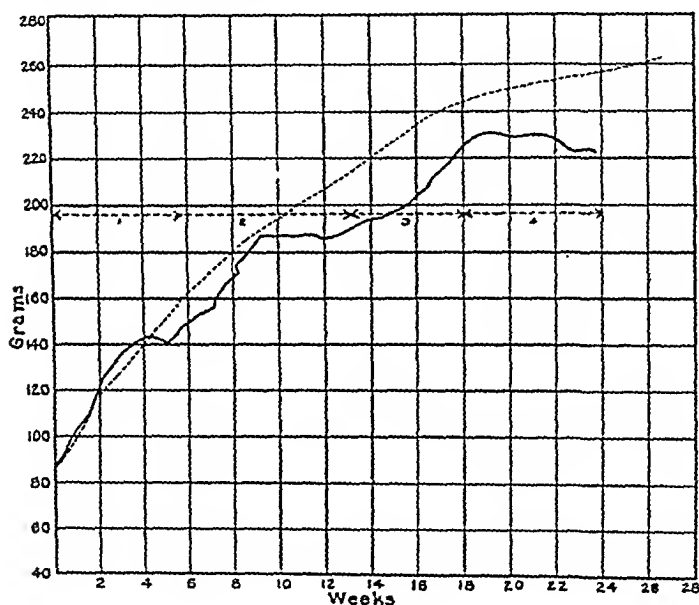


CHART I. *Rat 104* (male) shows the record of a rat which grew in a nearly normal manner during sixty-five days on a ration of purified food substances. The broken line represents the normal curve of growth. A suspension of growth occurred at this time and after four weeks without increase in body weight, 1 gram of ether extract of egg was added to the ration every other day, with the result that the animal gained 35 grams during the following forty-two days. The rations employed during the four periods of the experiment were as follows:

PERIOD I. per cent	PERIOD II. per cent	PERIOD III. per cent	PERIOD IV
Salt mixt..... 6	Salt mixt.... 6	Salt mixt.... 6	Same as III but
Casein..... 18	Casein..... 18	Casein..... 18	without the
Lard..... 20	Lactose..... 15	Dextrine.... 74	ether extract
Lactose..... 20	Dextrine.... 59	Agar-agar... 2	of egg.
Starch..... 31	Agar-agar... 2	Ether extract of	
Agar-agar.... 5		egg, 1 gm. every	
		other day.	

The salt mixture employed in these rations consisted of:

	grams		grams
Calcium lactate.....	3.785	Dipotassium phosphate....	3.648
Sodium citrate (anhydrous)..	3.296	Sodium chloride.	3.430
Magnesium citrate(10.2% Mg)	1.298	Ferric citrate.....	1.000
Potassium citrate.....	3.118		

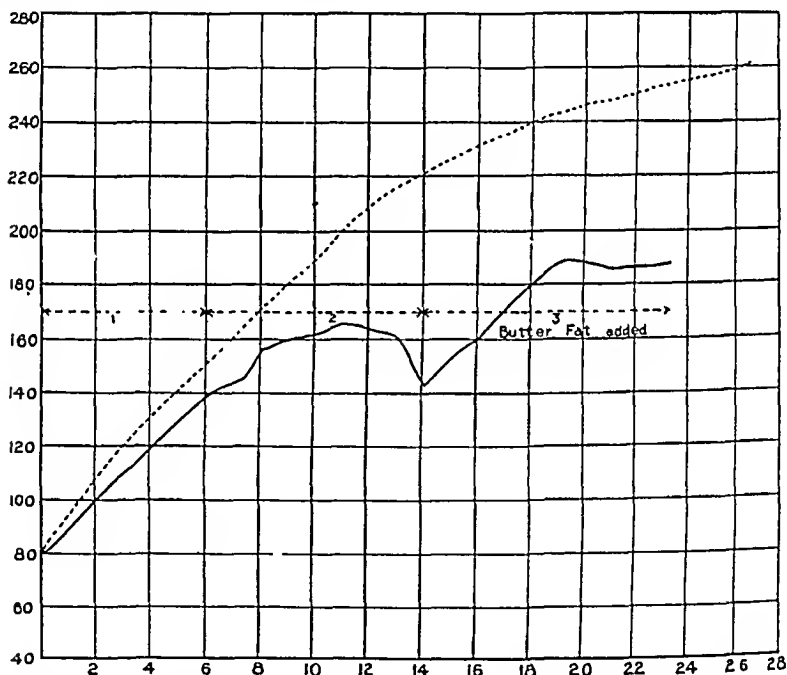


CHART II. *Rat 141* (male) shows the record of a rat which grew continuously although slightly under normal rate during eighty days on a ration of purified food substances. There was at this time a complete suspension of growth and a rapid decline in body weight. The addition of 10 per cent of ether-soluble butter fat to the diet led to a prompt resumption of growth during the following thirty-five days, when the rat gained 50 grams.

The rations employed were as follows:

PERIOD I.	per cent	PERIOD II.	per cent	PERIOD III.
Salt mixture.....	6	Salt mixture.....	5	Same as II with butter
Casein.....	12	Casein.....	12	fat replacing part of
Lard.....	20	Lactose.....	20	dextrine.
Lactose.....	15	Dextrine.....	61	
Starch.....	42	Agar-agar.....	2	
Agar-agar.....	5			

The salt mixture employed consisted of:

	grams		grams
Sodium chloride.....	0.61	Calcium lactate	11.38
Dipotassium phosphate.....	17.00	Magnesium citrate(10.2%Mg)	23.42
Monocalcium phosphate.....	1.63	Ferric citrate.....	1.00

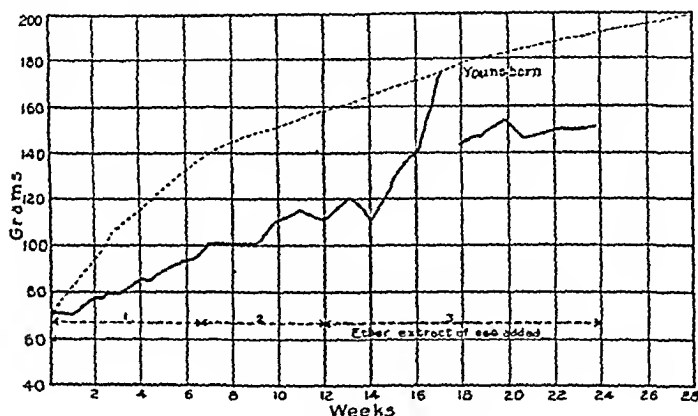


CHART III. *Rat 138* (female). This rat received a salt mixture which has induced much better growth in other rats. Her growth was but little over half normal during the first eighty-five days on a ration of purified food substances. She was with a male during this time but did not become pregnant. From the 86th day she was given 1 gram of ether extract of egg every other day. Growth was at once resumed and she became pregnant on the 14th day after the addition was made. On the 119th day of "artificial" feeding she gave birth to eight young which she suckled normally during twenty days. The aggregate weight of the young at the age of twenty days was 162 grams, the weight of the mother at this time being 155 grams. She is still in an excellent nutritive condition after one hundred and sixty-eight days and is continuing on the diet.

The rations employed in feeding this rat were as follows:

PERIOD I.	PERIOD II.	PERIOD III.	PERIOD IV.
per cent	per cent	per cent	per cent
Casein..... 18	Casein..... 18	Casein..... 25	Casein..... 18
Lard..... 25	Lactose..... 10	Lactose..... 20	Lactose..... 20
Lactose..... 10	Starch..... 62	Dextrine..... 40	Dextrine..... 40
Starch..... 37	Agar..... 5	Agar..... 5	Agar..... 2
Agar..... 5	Salt mixt.... 5	Salt mixt.... 10	Ether extract
Salt mixt.... 5	Ether extract		of egg and
	of egg add-		of butter.... 10
	ed.		Salt mixt..... 8

There have been many slight modifications in the character of the inorganic content of the rations of this rat.

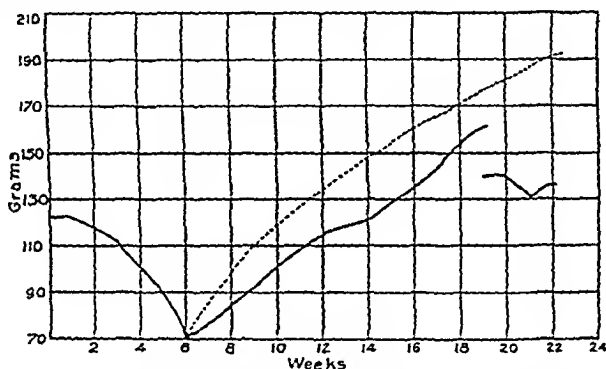


CHART V. *Rat 50* (female) is introduced to show what degree of success can be attained on diets containing no fats and but insignificant amounts of ether-soluble substances. The experiment covered one hundred and fifty-four days. During the first forty-two days the ration contained lard. There was a steady and rapid decline due apparently to the unsatisfactory character of the salt mixture fed, for on changing this there was a rapid recovery and nearly normal growth with the production of young after ninety-one days on a fat-free ration. The experiment was terminated because the mother ate two of her young and the third was only about half normal size on the 18th day (weight 10 grams). This one grew normally when placed with another mother suckling young of the same size.

The ration consisted of:

PERIOD I.		PERIOD II.			
	per cent		per cent		grams
Casein.....	18	Casein.....	18	NaCl.....	1.022
Lard.....	25	Lactose.....	20	MgSO ₄ (anhydrous)	3.865
Starch.....	33.5	Dextrine.....	51	Mg citrate.....	7.000
Lactose.....	10	Agar-agar.....	5	K ₂ HPO ₄	0.168
Agar-agar.....	5	Salt mixt. used in		KH ₂ PO ₄	12.795
Salt mixt.....	7.5	period I, Chart			
		II.....	6		

The salt mixture employed in period I consisted of:

	grams		grams
Na ₂ HPO ₄ 12H ₂ O.....	5.052	CaH ₄ (PO ₄) ₂ H ₂ O.....	1.880
Na citrate (anhydrous).....	2.866	CaSO ₄ (anhydrous).....	0.168
Calcium lactate.....	25.377	Ferric citrate.....	1.000

We have seen this prompt resumption of growth after a period of suspension result from the introduction of ether extract of butter or of egg in about thirty animals and are convinced that these extracts contain some organic complex without which the animals cannot make further increase in body weight, but may maintain themselves in a fairly good nutritive state for a prolonged period. In no instance have we obtained such a result by the feeding of lard, or of olive oil. It is therefore not merely the absence of fats from the diet which causes the suspension of growth.

Whether the resumption of growth is the result of supplying in the ether extract of egg or of butter, some indispensable organic complex of the chemical nature of the lipins, or is the result of a stimulating action of some substance accompanying the lipins, cannot be decided from the data at present available. In a considerable number of instances we have fed lecithin or cholesterol with very doubtful results. Hopkins⁵ observed that small quantities of milk added to rations of purified food substances, exert an influence on the growth of rats, which is out of all proportion to the added nitrogen or calorific value. Funk⁶ and Suzuki, Shimamura and Odaki have isolated substances from rice polishings which exert a remarkable curative effect on birds suffering from polyneuritis as a result of exclusive feeding on polished rice.

⁵ Hopkins: *Journ. of Physiol.*, xliv, p. 425, 1912.

⁶ Funk: *Journ. of Physiol.*, xlv, p. 50, 1912; Suzuki, Shimamura and Odaki: *Biochem. Zeitschr.*, xliii, p. 89, 1912.

Funk has also obtained preparations from brain, yeast, and milk which have the same power.

The extensive literature on the remarkable physiological properties of certain fresh food-stuffs as contrasted with the cooked or preserved materials, in preventing or curing scurvy and beriberi, diseases arising from unsatisfactory diets, has been recently summarized by Cooper.⁷ From the experimental data available it seems apparent that very young animals cannot be made to complete their growth on rations supplying only purified proteins, carbohydrates, fats, and salts. Our observation that ether extracts from certain sources improve the condition of animals on such rations, strongly supports the belief that there are certain accessory articles in certain food-stuffs which are essential for normal growth for extended periods.

It is interesting in this connection to correlate our observation on the physiological properties of ether extracts of butter or eggs, with those of Osborne and Mendel on the power of an animal to maintain itself on a ration containing gliadin as the only protein. While no growth is possible on this ration, notable increase in weight due to the building of young, can take place and a milk supply capable of normally nourishing the young can be produced. Through the agency of the ovary in egg production, or the mammary glands in milk production, the necessary accessory bodies essential to the proper nourishment of the young are readily synthesized by the animal cell. The young themselves have not the power to produce these syntheses for their own preservation when these unknown substances are lacking in the diet.

The further study of the nature of the "active" bodies in these extracts must of necessity require a great deal of time and labor, since preparations from butter or eggs made with solvents poorer than ether, and of ether extracts from other sources, the examination of their stability, etc., can be tested only on animals which have been grown as far as possible on rations of purified food substances, and have reached the stage of suspension of growth.

This work will be carried on as rapidly as circumstances will permit.

⁷ Cooper: *British Med. Journ.*, No. 2727, p 722, 1913.

THE BIOCHEMICAL RELATION BETWEEN PYRUVIC ACID AND GLUCOSE.

By H. D. DAKIN AND N. W. JANNEY.

(From the Herter Laboratory and the Chemical Laboratory of the Montefiore Home, New York.)

(Received for publication, June 1, 1913.)

In a recent communication by Paul Mayer¹ an unsuccessful attempt was made to demonstrate glucose synthesis from pyruvic acid in phlorhizinized animals. These negative results were the more surprising on account of Paul Mayer's earlier investigations² upon the effect and fate of pyruvic acid in the animal body. In these experiments it was shown in the most convincing fashion that glucose may be excreted in the urine on administering pyruvic acid to rabbits, that this effect is accompanied by hyperglycaemia, and finally that glycogen synthesis could be shown to follow consumption of pyruvic acid by previously starved rabbits.

As a result of his negative experiments Paul Mayer is inclined to the belief that a definite proof of the formation of glucose from pyruvic acid cannot be furnished by the use of glycosuric animals. Our own experiments, which were completed before the publication of Mayer's second paper, lead to an entirely different conclusion. Moreover, we have learned from conversation with Dr. Ringer that he has obtained results essentially similar to ours. By mutual arrangement Dr. Ringer's results appear simultaneously with our own (p. 145).

We find that the sodium salt of pyruvic acid given by mouth to diabetic animals under suitable conditions may give rise to almost as large an excretion of "extra glucose" as does administration of lactic acid itself. In these experiments we have used dogs treated with phlorhizin and also human diabetics. When sodium pyruvate is administered subcutaneously to glycosuric

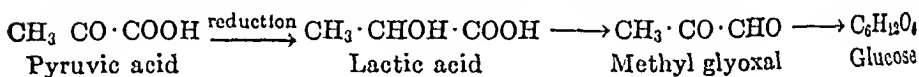
¹ *Biochem. Zeitschr.*, xlix, p. 486, 1913.

² *Ibid.*, xl, p. 441, 1912.

dogs, especially to small animals, a definite but relatively small increase in "extra glucose" is noted. Incidentally we have observed that pyruvic acid which has undergone extensive polymerization on long standing may yield little or no glucose. It is therefore important for these experiments that the acid be freshly distilled before use.

A consideration of our results together with those of Paul Mayer leads us to the belief that glucose cannot be formed directly from pyruvic acid but that if the conditions are favorable for the reduction of the latter substance to lactic acid, then glucose may be produced. Such a hypothesis would harmonize with our observed experimental data and would be in entire accord with a hypothesis concerning glucose formation recently put forward by one of us in conjunction with Dudley.³ It should be noted in this connection that Mayer has actually detected inactive lactic acid in the urine of normal rabbits receiving sodium pyruvate, and it is now known that both *d*- and *l*-lactic acids and methyl glyoxal may lead to the synthesis of glucose.

Our idea of the relationship between pyruvic acid and glucose may be gathered from the following scheme:



If the experimental conditions do not favor the initial reduction of pyruvic acid to lactic acid, it is probable that no synthesis of glucose can follow.

The experimental results are contained in the following tables. The general conditions were similar to those described in previous publications. The phlorhizin was given suspended in olive oil according to Coolen's method. In every case the excretion of acetoacetic acid and β -hydroxybutyric acid was followed, but no increase was observed to follow the administration of pyruvic acid. The figures are therefore omitted.

³ This *Journal*, xiv, p. 555, 1913.

EXP.	PERIOD	GLUCOSE	NITROGEN	G:N	EXTRA GLUCOSE	SUBSTANCE GIVEN
I	I			3.28		
	II	11.42	3.48	3.31		
	III	19.53	3.38	5.78	8.75	12.5 gms. pyruvic acid.
	IV, V	9.81	3.15	3.11		
	VI	16.56	3.21	5.16	8.46	10.7 gms. lactic acid.
	VII	12.15	3.03	3.94		
II	I	25.38	7.83	3.24		
	II	27.03	7.83	3.45		
	III	25.07	6.43	3.88	3.40	12 gms. pyruvic acid.
	IV	25.91	7.60	3.41		
	V	29.92	7.29	4.14	5.35	12 gms. pyruvic acid.
III	I	28.19	8.93	3.16		
	II	32.15	8.12	3.96	6.65	10 gms. pyruvic acid.
	III	27.52	8.82	3.12		
	IV	26.29	8.91	2.95	0	15 gms. polymerized pyruvic acid.

EXP.	PERIOD	GLUCOSE	NITROGEN	CARBOHY- DRATE IN DIET AS GLUCOSE	SUBSTANCE GIVEN
IV	I	4.39	11.56	13.2	
	II	8.28	12.25	16.8	
	III	5.73	9.90	16.1	
	IV	7.12	10.43	17.7	
	V	21.30	11.72	17.0	44 gms. pyruvic acid in 5 portions.
	VI	21.20	15.34	19.7	
	VII	5.72	10.97	17.9	
	VIII	5.92	10.22	17.7	
	IX	20.37	9.37	17.9	37 gms. lactic acid in 7 portions.
	X	18.55	8.11	16.3	
	XI	12.75	11.52	18.4	

EXPERIMENT I. Dog weighing 12 kgms. Sodium pyruvate and lactate given by stomach tube. Urine collected in 6-hour periods. Using $G:N = 3.22$ it may be calculated that 12.5 grams of pyruvic acid gave 8.75 grams "extra glucose," while 10.7 grams lactic acid gave 8.46 grams.

EXPERIMENT II. Bitch weighing 8 kgms. Urine collected in 12-hour periods. Sodium pyruvate given subcutaneously in period II, by mouth, in period V. Using $G:N = 3.37$, the subcutaneous administration of 12

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grams of pyruvic acid appears to give 3.4 grams of "extra glucose," while the same amount given by mouth yielded 5.35 grams.

EXPERIMENT III. Dog weighing 19 kgms. 10 grams of pyruvic acid as sodium salt given by mouth gave 6.65 grams of "extra glucose." 15 grams of polymerized pyruvic acid given subcutaneously led to no additional glucose excretion.

EXPERIMENT IV. Female. Moderate case of diabetes mellitus. Multiple sclerosis and slight tubercular involvement of right pulmonary apex. During a preliminary period of nine days when the total carbohydrate consumption averaged 80 grams per day, the average daily glucose excretion was 32 grams. A large increase in glucose excretion is seen to follow the administration of both sodium pyruvate and lactate.

EXPERIMENT V. A mild case of diabetes mellitus, similar to the above. Only a trifling increase (2-7 grams) in glucose excretion followed the consumption of sodium pyruvate (28 grams) and sodium lactate (5.3 grams). The analyses show little of interest and are not reproduced.

THE RELATION OF DIETS AND OF CASTRATION TO THE TRANSMISSIBLE TUMORS OF RATS AND MICE.

By J. E. SWEET, ELLEN P. CORSON-WHITE AND G. J. SAXON.

(From the Laboratory of the American Oncologic Hospital, Philadelphia, Pa.)

(Received for publication, June 3, 1913.)

The study of the transmissible tumors of animals has brought to the attention of every worker in this field, facts of curious variations in the reaction to the tumor of animals obtained from different sources. A transmissible tumor of the mouse, for instance, can be brought to a quite constant degree of virulence, as measured both by the percentage of positive inoculations and by the rate of growth, on a given lot of mice; but mice obtained from a different source may show a marked diminution in the number of "takes" and an increase in the number of retrogressions. After a series of inoculations in the new strain of mice, especially if previous to inoculation the mice have been subjected to the same conditions as the animals in which the standard of virulence had been determined, the virulence can gradually be brought back to the original standard.¹ Haaland² found, for example, that a tumor which gave 100 per cent takes in Berlin mice, gave only 24 per cent in Hamburg mice, and was practically harmless in Christiana mice. An apparently related phenomenon is noted in the so-called spontaneous tumors of mice; a breeder may find a large number of tumors among his animals but they can only rarely be successfully transplanted.

A large number of the subcutaneous adenocarcinomata of mice are not transmissible even into mice of the same strain, while other tumors of similar histological structure are transplanted easily.³ In all cases the transmissible tumor is most easily transplanted to the original tumor-bearing animal and next to animals

¹ Cuénot and Mercier: *Compt. rend. de l'Acad. des Sci.*, cxlvii, p. 1003, 1908.

² Haaland: *Berl. klin. Wochenschr.*, xlv, p. 713, 1907.

³ Loeb: *International Clinics*, p. 121, 1907.

of the same strain. Perhaps the most striking case of this peculiarity of tumors is recorded in the work of Rous,⁴ with a transmissible tumor of the fowl. This tumor, found in a pure blood Plymouth Rock hen was at first only transferable to birds of precisely the same strain as that in which the original tumor occurred, indeed only to birds from the same brood as the original tumor fowl.

These facts have naturally engaged the attention of many workers, and numerous studies have been made of the factors which might be expected to influence this condition of susceptibility or immunity of the host. Loeb⁵ finds that little if any influence seems to be exerted by the sex of the animal on the results with the transplantable tumors, while the spontaneous tumors of mice are found almost exclusively among the females.⁶ Haaland,⁷ Cuenot and Mercier,⁸ Uhlenhuth and Weidanz⁹ all state that the growth of a neoplasm is retarded during pregnancy and may even cease. This retardation has been thought to be due to the tax on the mother during gestation, and to be comparable to the unfavorable soil for tumor inoculation found in sick or ill-nourished animals.¹⁰ This explanation is however probably not true, for under normal conditions pregnancy is a stimulus to growth and to nutrition.¹¹

The age of the animal seems to exert a certain influence in so far that animals one-half to three-quarters grown seem to be the most favorable hosts for the transplantation of tumors.¹² The factor of heredity has been studied but the results are not very definite as yet.¹³ We have been able to make a few observations

⁴ Rous: *Journ. of Exp. Med.*, xiv, p. 696, 1910.

⁵ Loeb: *loc. cit.*

⁶ Jobling: *Monograph of Rockefeller Institute*, i, p. 174, 1910.

⁷ *Loc. cit.*

⁸ Cuénot and Mercier: *Compt. rend. soc. biol.*, lxvii, p. 736, 1909.

⁹ Uhlenhuth and Weidanz: *Arbeit. a. d. Kais. Gesundheitsamte*, xxx, p. 434, 1909.

¹⁰ Joannovics: *Wien. klin. Wochenschr.*, 1912, p. 36.

¹¹ Rous: *Journ. of Exp. Med.*, xiii, p. 248, 1911.

¹² Bradford, Murray, Haaland and Bowen: Third Scientific Report, Imperial Cancer Research Fund, 1900, p. 265.

¹³ Tyzzer: *Journ. of Medical Research*, xxi, p. 519, 1909; Cuénot and Mercier: *Compt. rend. soc. biol.*, lxi, p. 645, 1910; Loeb and Fleischer: *Zentralbl. f. Bakt.*, lxvii, p. 3, 1912.

upon the relation of heredity to the rat tumor, through the generous coöperation of the Wistar Institute. Our rats have been discarded litters from certain breeding experiments conducted by the Wistar Institute, and these litters have been kept as units in our work. The susceptibility or immunity has been, almost invariably, not a matter of individual animals but of the entire litter; the degree of susceptibility has also proven to be a question of the litter, not of the individual. For example, the same tumor may not take at all in one litter, prove slightly virulent for a second litter as measured by the rate of growth, and extremely virulent for a third litter. The suggestion to make use of the litter unit was offered to us by Dr. H. H. Donaldson, and, while our observations on this line are not complete, we are inclined to agree with those of larger experience in breeding experiments, that observations of litter units may in tumor work also, prove of greater value than observations of large numbers of mixed animals.

A few investigators have experimented to some extent upon the relation of diet to the growth of transplantable tumors. Up to the time of the beginning of our work, however, the literature contained but few such references and the experiments described seem to have been of a somewhat desultory character. Jensen¹⁴ suggested that diet might possibly influence the recurrence and metastases. Haaland¹⁵ discusses the effect of light, heat, moisture, etc., and concludes that diet is probably of the most importance. He found that mice on a diet of hemp seed, bread and milk, with some oats, were more susceptible to a certain sarcoma than those on ordinary diet; while those animals limited to a diet of bread and oats were the least susceptible. Stahr¹⁶ studied the effect of diet on a home strain of mice and a resistant strain from another locality, finding that a diet of hemp seed and milk rendered the animals less susceptible than the eating of bread and water. Moreschi¹⁷ made careful studies on diet with reference to the changes in weight of mice and the rate of growth of

¹⁴ Jensen: *Zeitschr. f. Krebsforschung*, xx, p. 682, 1909.

¹⁵ Haaland: *loc. cit.*

¹⁶ Stahr: *Centralbl. f. allg. Path. u. path. Anat.*, xx, p. 628, 1909.

¹⁷ Moreschi: *Zeitschr. f. Immunitätsforschung*, etc., ii, p. 651, 1909.

their tumors, as did Medigreceanu.¹⁸ The unfavorable influence of poor nutrition as brought about by intercurrent disease upon the rate of growth of the transplanted tumor is a matter of general observation. Moreschi¹⁹ found that in animals with the food supply reduced to a minimum compatible with life, with no evidence of previous or obvious illness, tumors develop less frequently and grow more slowly than in the controls. Our own work along the line of the influence of diet was based upon the work of Mendel and Osborne,²⁰ who found in their studies of the effects of feeding rats with combinations of pure vegetable proteins a number of diets which completely retarded the normal growth of the animal, although the general condition seemed entirely normal. In other words their animals were not starved in any sense except a very specific one—certain elements necessary to normal growth were lacking. It is not the place in this paper to discuss their findings of such importance to our knowledge of nutrition and growth. We gladly accepted their work as an opportunity to study this relation of nutrition and growth to a transplanted tumor. To put the question more concisely, regardless of whatever may be the ultimate cause of the cancer growth, could a cancer grow in a body rendered incapable of normal cell growth?

In our experiments we systematically used one-half or three-quarter grown rats and mice; two weeks were allowed to pass between the beginning of the special diet or the operative interference and the inoculation of the animal. The control diet was made up of bread, oats, wheat, rice, corn, sunflower seed and water. The special diet was made up on a basis of the diet used by Mendel and Osborne for rat 179.²¹ Preliminary experiments showed that a gluten mass obtained by careful washing of wheat flour could be satisfactorily used in place of the chemically pure substances studied by Mendel and Osborne. By this we mean that the animals could be kept at an almost constant weight—some indeed absolutely constant—for the periods of time neces-

¹⁸ Medigreceanu: *Berl. klin. Wochenschr.*, xlvii, p. 772, 1910; *Proc. Royal Society*, lxxxii, p. 286, 1910.

¹⁹ *Loc. cit.*

²⁰ Mendel, Osborne and Ferry: *Zeitschr. f. physiol. Chem.*, lxxx, p. 307, 1912.

²¹ *Zeitschr. f. physiol. Chem.*, lxxx, p. 318, 1912.

sary for tumor work. We have no doubt that our results would have been more marked had we made use of chemically pure glutenin and gliadin, but since the gluten in our hands effectively prevented growth and because of the difficulty of obtaining a sufficient quantity of chemically pure substances for feeding large series of animals we felt obliged to content ourselves. The diet was therefore made up of gluten 18 per cent, starch 24.5 per cent, lard 27 per cent, lactose 23 per cent, salt mixture 2.5 per cent, agar 5 per cent.

The original tumors used in our work were obtained from the Rockefeller Institute through the courtesy of Dr. Peyton Rous and were the Flexner-Jobling adenocarcinoma of the rat, and the Rockefeller Institute mouse tumor No. 33, a carcinoma. In every experiment bits of the same tumor were used for the inoculation of the controls and the animals under special treatment.

TABLE I.

DIET	NUMBER OF MICE	NUMBER OF TAKES	LATENT PERIOD IN DAYS	AVERAGE DIAMETER IN MILLIMETERS OF TUMORS AFTER 16 DAYS
Normal.....	25	23	7	2.2
Mendel-Osborne.....	25	9	9	0.9

At the end of sixteen days this experiment was divided as follows: the nine positive animals on the Mendel-Osborne diet were continued on this diet; four days later, or twenty days after inoculation, five of these nine tumors had apparently completely retrogressed. Ten days later, or thirty days after inoculation, two of the remaining four had retrogressed. One of the two mice left died on the fifty-second day with a tumor measuring 5.2 mm. in its largest diameter. The remaining mouse on the fifty-second day had a tumor 4 mm. in diameter. It was then changed to a normal diet, and at the end of thirty days was possessed of the tumor shown in the photographs on the following page.

The sixteen negative mice of this series were changed at the end of sixteen days to a normal diet; three developed tumors five days later or twenty-one days after inoculation. Ten days



later or twenty-six days after inoculation these tumors had a diameter of 1.7 mm.; twenty days later or thirty-six days after inoculation 7 mm., and thirty days later or forty-six days after inoculation, 21 mm. The latent period of twenty-one days in these animals is noteworthy. The 23 mice of the normally fed group were divided as follows:

TABLE II.

DIET	NUMBER OF MICE	SIZE OF TUMOR IN MILLIMETERS AFTER INOCULATION			RETROGRESSIONS
		16 days	20 days	30 days	
Normal.....	11	2.2	4.8	19.7	3
Mendel-Osborne.....	12	2.2	3.4	5.1	5

This experiment was repeated by inoculating one hundred mice, fifty males and fifty females, with bits of the same tumor and dividing them as follows:

TABLE III.

DIET	NUMBER OF MICE	TAKES	LATENT PERIOD IN DAYS	AVERAGE DIAMETER OF TUMOR IN MILLIMETERS, AFTER		
				10 days	20 days	30 days
Normal.....	25 males	19	7	6.7	15.9	19.3
Normal.....	25 females	15	7	4.5	9.2	15.7
Mendel-Osborne.....	25 males	7	12		2.4	5.3
Mendel-Osborne.....	25 females	8	10	1.0	3.1	8.4

The results with the rat tumor have been of the same general trend; as others have observed, however, work with the rat tumor is less satisfactory than with the mouse tumor, the rat tumor showing many unexplainable irregularities in its behavior. Because of this irregularity of behavior we have included in the following table only those rats on the Mendel-Osborne diet in which there was a definite "take."

TABLE IV.

DIET	NUMBER OF RATS	TAKES	LATENT PERIOD	AVERAGE DIAMETER IN MILLIMETERS AFTER			RETROGRESSIONS
				10 days	20 days	30 days	
Normal.....	59	31	9 days	6.1	9.8	14.7	0
Mendel-Osborne....	59	31	9 days	4.9	7.2	10.0	8

It would seem fair to conclude from these experiments that the number of successful transplantations can be markedly reduced by a diet which prevents normal body growth. The rate of growth of the tumor is much slower and the number of retrogressions is high on such a diet. The effect of diet would seem to be more marked upon the receptivity of the host, as shown in the diminished number of takes and the increase of latent period seen in Table III, and yet that this diet can influence a tumor which has already started is seen in Table IV where nearly 25 per cent of retrogressions occurred on a special diet, with no retrogressions in the controls.

We have further experimented with the diets described by Reid Hunt²² as influencing the thyroid gland. An exclusive diet of

²² Reid Hunt: Hygienic Laboratory Bulletin 69, 1910.

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oats and water or 2 per cent KI solution is supposed to accelerate the thyroid function, a fact in line with the findings of Watson,²³ who found the thyroid enlarged in young rats fed wholly on oats. The second diet, eggs and milk, is said to retard or inhibit thyroid function. This diet however proved to be so detrimental to the health of the animals, both rats and mice, that only a very few were able to thrive; these few, after a preliminary diarrhoea, grew fat and seemed to be in the best of health.

TABLE V.

DIET	NUMBER OF EXPERIMENTS	NUMBER OF MICE	TAKES	LATENT PERIOD IN DAYS	AVERAGE DIAMETER IN MILLIMETERS OF TUMORS AFTER		
					10 days	20 days	30 days
Normal.....	14	250	103	7	2	7	10
Thyroid accelerating...	14	250	166	7	4	12	27
Thyroid inhibiting.....	5	75	12	12		4	7

TABLE VI.

DIET	NUMBER OF EXPERIMENTS	NUMBER OF RATS	TAKES	LATENT PERIOD IN DAYS	AVERAGE DIAMETER IN MILLIMETERS OF TUMORS AFTER	
					20 days	30 days
Normal.....	7	74	52	14	6.1	27.0
Thyroid accelerating ..	3	50	36	10	7.4	31.4
Thyroid inhibiting.....	3	26	10	15	4.0	15.0

Whether or not the interpretation of the experiments from which it was concluded that the thyroid gland is influenced by these diets be correct, we may at least conclude that here again as with the Mendel-Osborne diets the transplantable tumor can be influenced by the diet. It is in accord with our idea that the influence of diet may be exerted through the ductless glands, and it was from this point of view that we made the experiments described above.

Our next studies were undertaken with the specific purpose of studying the effect of the removal of certain of the ductless glands

²³ Watson: *Lancet*, i, p. 985, 1907.

upon the transplanted tumor. Our work has been limited to castration of the male animals chiefly because of our knowledge of the work of Dr. Hatai (now in press from the Wistar Institute) upon the relation of castration to the other ductless glands. Hatai finds after castration a persistence of the thymus and an increase of 74 per cent in the weight of the hypophysis. Studies of the effect of castration upon tumors have been made before. Graf²⁴ made five experiments using sixty-six castrate males, forty normal males, fifty-six castrate females, and thirty-six normal females, and inoculating with the most malignant tumor in his possession. He concludes that there is no essential difference in the tumor takes or in the energy of growth between the normal and the castrated animals. It seems to us that his experiment was hardly properly designed to bring out any differences, since changes in the nature of an increase of susceptibility might be masked by using a tumor which gives 100 per cent of positive inoculations in the controls. Rohdenburg, Bullock and Johnston²⁵ removed the thyroid, thymus, spleen and testes, and inoculated after varying intervals with a tumor. Their results after castration show only a marked shortening of the latent period.

TABLE VII.

	NUMBER OF EXPER- MENTS	NUMBER OF ANIMALS	TAKES	LATENT PERIOD IN DAYS	AVERAGE DIAMETER IN MILLIMETERS OF TUMORS AFTER		
					10 days	20 days	30 days
Normal mice	3	73	30	7	1.8	5.0	12.0
Castrated mice.....	3	73	54	7	2.0	16.0	31.0
Normal rats.....	2	15	5	10	4.0	8.0	18.0
Castrated rats	2	14	9	10	7.0	14.0	47.0

It would seem from our experiments that the removal of the testes in some way renders an animal a more receptive host as seen by the increased takes and the more rapid growth.

In order to make a more satisfactory comparison of the effects of the diets and of castration we did the following two experiments, the results of which are embodied in Table VIII. Two

²⁴ Graf: *Centralbl. f. Path.*, xl, p. 17, 1909.

²⁵ Rohdenburg, Bullock and Johnston: *Arch. of Internal Med.*, vii, p. 491, 1911.

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series of 150 mice in each series, each series of 150 mice being inoculated with the same tumor, were divided as seen in the table.

TABLE VIII.

	NUMBER OF MICE	TAKES	PER CENT OF TAKES	LATENT PERIOD IN DAYS	AVERAGE DIAMETER IN MILLIMETERS OF TUMORS AFTER		
					10 days	20 days	30 days
Normal diet.....	75	56	74.6	7	2.4	8.1	22.8
Mendel-Osborne diet ..	75	18	24.0	14		2.1	4.8
Thyroid accelerating ..	50	40	80.0	7	3.1	9.4	28.9
Thyroid inhibiting	16	2	12.5	17		3.8	5.3
Castrates.....	50	44	88.0	7	4.9	17.4	43.1

Certain facts of clinical experience formed the basis of the work outlined in this paper. We know for instance of the relation of the thyroid to growth, as seen in cretinism; of the relation of the pituitary to acromegaly and gigantism and of the control of the pituitary over the only instance of embryonic cell reproduction which persists in adult life, spermatogenesis and oögenesis. Finally it is a clinical fact that cancer notwithstanding many exceptions is a disease of the menopause, that period of life when certain of the ductless glands lose their normal function, this loss entailing related changes in the whole chain of the interrelated functions of the ductless glands. A further thought was that such a study might finally lead to some light on the question of the parasitic nature of the disease, for it would seem difficult to assume that a parasitic process would obey the laws of normal growth. Finally, as in tuberculosis, it may be found with cancer that the best treatment will be one not directed primarily against the disease process, but directed toward the stimulation of the normal protective functions of the body.

It may very fairly be objected that in no instance, except perhaps that of castration, in which our results do not entirely agree with those found by others, have we shown that any influence of our experiments is in fact brought about through the function of the ductless glands. We grant this objection and will simply leave our hypothesis stand for the present, since it at least shows that our work has been directed toward a definite goal. In what-

ever manner it is to be explained, we conclude from our experiments that:

1. The susceptibility of rats and mice to the transplantable tumors may be influenced both positively and negatively by proper diets.

2. The rate of growth of the transplanted tumors can be positively or negatively influenced by proper diets.

3. Castration of the male renders the animal more receptive to the transplanted tumor and the rate of growth of the tumor is increased.

ON CEREBRONIC ACID.

THIRD PAPER.

ITS BEARING ON THE CONSTITUTION OF LIGNOCERIC ACID.

By P. A. LEVENE AND C. J. WEST.

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(Received for publication, June 9, 1913.)

In our study of cerebronic acid¹ an acid of the same composition and melting point as lignoceric acid was obtained by the action of alkaline potassium permanganate upon the potassium salt of cerebronic acid. It was pointed out at the time that this acid might be identical with lignoceric acid. Since then we have prepared derivatives of this new acid, which prove to be the same as those given in the literature for lignoceric acid.²

It has been shown that cerebronic acid has a normal carbon chain by reduction to pentacosan, melting at 53°-54°. This was further confirmed by the isolation of a small amount of a hydrocarbon melting at 51°-52° from the reaction product of hydroiodic acid upon the new acid, $C_{25}H_{50}O_2$. Thus it is quite definitely established that the new acid has a normal carbon chain. Since this acid is identical with lignoceric acid, then lignoceric must be considered a normal acid of twenty-four carbon atoms.

The following gives a comparison of the acids and their derivatives:

	Lignoceric acid	Acid from Cerebronic acid
	m. p.	m. p.
Acid.....	80.5°-81°	80°-81°
Methyl ester.....	55°	55°
Ethyl ester.....	57°-58°	58°
Lead salt.....	117°	116°-117°

¹ Levene and Jacobs: this *Journal*, xii, p. 381, 1912; Levene and West: *ibid*, xiv, p. 257, 1913.

² Hell and Hermann: *Ber. d. deutsch. chem. Gesellsch.*, xiii, p. 1713, 1880; Kreiling: *ibid*, xxi, p. 880, 1883.

EXPERIMENTAL.

Methyl ester.

The methyl ester of the new acid was prepared by boiling 2 grams of the acid, 100 cc. of absolute methyl alcohol and 4 cc. of concentrated sulphuric acid four hours on the water bath. The ester which separated on standing over night at 0°, was recrystallized from methyl alcohol twice, then from acetone and finally dried in the chloroform bath for two hours. It melted at 58°. Hell and Hermann give 56.5°–57°; Kreiling, 58°.

0.1200 gram of substance gave 0.3450 gram CO₂ and 0.1416 gram H₂O.

	Calculated for C ₂₄ H ₄₀ O ₂ .CH ₃ :	Found:
C.....	78.53	78.41
H.....	13.09	13.20

Ethyl ester.

The ethyl ester was prepared in the same way as the methyl ester. It was recrystallized from ethyl alcohol three times, when it melted at 55–56°. It was then evaporated to dryness with a slight excess of sodium methylate and extracted with ether to remove any trace of free acid and recrystallized from acetone, when it melted at 55°. Both Hell and Kreiling give 55° as the melting point of the ethyl ester.

0.1194 gram of substance gave 0.3386 gram CO₂ and 0.1408 gram H₂O.

	Calculated for C ₂₄ H ₄₀ O ₂ .C ₂ H ₅ :	Found:
C.....	78.79	79.12
H.....	13.13	13.20

Lead salt.

The lead salt was prepared by treating a hot methyl alcohol solution of the acid with lead acetate in the same solvent as long as a precipitate formed. A drop of ammonia was then added to complete the precipitation. This was filtered off when cool, washed with warm methyl alcohol and dried in vacuum. When heated quickly it softened at about 110° and melted at 116°–117°. Hell and Hermann give the melting point as 117°.

0.1200 gram substance gave 0.2694 gram CO_2 and 0.1073 gram H_2O .

	Calculated for $(\text{C}_{24}\text{H}_{57}\text{O})_2\text{Pb}$:	Found:
C.,.....	61.19	61.23
H.,.....	10.06	10.01

COLORIMETRIC DETERMINATION OF EPINEPHRINE IN DESICCATED SUPRARENAL GLANDS.

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(Received for publication, June 3, 1913.)

In a previous paper¹ from this laboratory it was shown that the color developed by heating desiccated suprarenal glands with a faintly acidified solution of potassium iodate could be used as an approximate measure of the epinephrine content of the sample, as determined by the blood pressure method. On the basis of the experiments then made, details were proposed for a rapid colorimetric estimation of the physiological activity of commercial desiccated suprarenal glands.

Although the iodate reagent was selected as the best of quite a number that were tried, it possessed the disadvantage of yielding slightly variable shades of color with different samples of glands. The method, therefore, could not be applied with equal success to all samples. In spite of this defect the approximate values that were obtained with nine samples of glands showed that even this more or less imperfect method could be made to serve a useful purpose, in differentiating between inferior samples and those of average physiological activity.

Since the publication of our paper, other and more delicate color reactions for epinephrine, have been proposed. Of these the phosphotungstic acid reagent of Folin and Denis² and the gold chloride reagent for which great sensibility has recently been claimed by Gautier³ are particularly worthy of attention.

¹ Hale and Seidell: *Amer. Journ. of Pharm.*, lxxxiii, pp. 551-8, (Dec.) 1911.

² Folin and Denis: this *Journal*, xii, p. 239, 1912; Folin, Cannon and Denis: *ibid.*, xiii, pp. 477-483, 1913.

³ Gautier: *Compt. rend. de la soc. de biol.*, lxxxiii, pp. 564-565, 1912.

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Phosphotungstic acid reagent of Folin and Denis. In their paper with Cannon the authors give minute details in regard to the estimation of the epinephrine in fresh suprarenal glands. It was, therefore, only necessary to introduce the modifications required for determinations in dried instead of the moist glands. The weights of moist glands which had been used varied from 0.164 to 11.69 grams, hence it was concluded that 0.5 gram of desiccated material would be a convenient amount to use. The determinations, the results of which are shown in table I, were made as follows: Fifteen cubic centimeters of $\frac{N}{10}$ hydrochloric acid and 0.5 gram of the sample of desiccated suprarenal gland were mixed and after about an hour, 45 cc. of water were added and the solution heated to the boiling point; 5 cc. of 10 per cent sodium acetate solution were then added and the boiling continued a minute or so; after cooling, the solution was diluted to 100 cc. and well mixed. To 5 cc. of the clear supernatant liquid, 2 cc. of the phosphotungstic acid reagent and 20 cc. of saturated sodium carbonate solution were added. The mixture was diluted to 100 cc., thoroughly mixed and the intensity of its blue color estimated by comparison in a Duboscq colorimeter with standards made simultaneously from 0.1 per cent uric acid solution.⁴

The phosphotungstic acid method as developed by its authors certainly has much to commend it. The fine blue color yielded by the uric acid standard and the gland samples appears to be of exactly the same shade and the relative intensities of different solutions can be accurately estimated. The sensitiveness of the reaction is such that the developed color is not sensibly modified by any extractive material derived from the sample itself, as appears to be the case with the iodate reaction. Therefore, disturbing differences of shades of color are not observed with different commercial samples. The exceptional sensitiveness also permits an apparent accuracy to the second and possibly even

⁴ In the epinephrine paper of Folin, Cannon and Denis (*loc. cit.*) the authors omit to state that alkali must be added to effect solution of the uric acid. In a previous paper by Folin and Macallum, Jr. (*this Journal*, xiii, p. 366, 1912), it is directed that 0.25 gram uric acid be dissolved with the aid of 25 cc. of 0.4 per cent Li_2CO_3 and the solution diluted to 250 cc. Following these directions I found that all of the uric acid did not dissolve in two and one-half hours and, therefore, added a second 25 cc. of Li_2CO_3 solution in order to obtain a clear solution.

TABLE I.

Percentages of epinephrine in commercial desiccated suprarenal glands as determined by the phosphotungstic acid method of Folin and Denis.

SAMPLE NO.	SOURCE	AVERAGE OF AT LEAST 5 READINGS IN DUBOSCQ COLORIMETER		CALC. GMS. EPINEPHRINE IN ALIQUOT USED	CALC. PER CENT EPINEPHRINE IN SAMPLE
		Sample	Standard		
362	Sheep	25	14.1	0.000188	0.752
363	Sheep	25	14.0	0.000187	0.748
365	Sheep	25	11.0	0.000147	0.588
366	Sheep	25	4.2	0.000056	0.224
367	Sheep	25	12.9	0.000172	0.688
368	Sheep	25	6.2	0.000083	0.332
369	Sheep	25	8.2	0.000109	0.436
370	Sheep	25	1.9	0.000025	0.100
416	Sheep	25	6.5	0.000087	0.348
417	Beef	25	17.2	0.000229	0.916
418	Hog	25	5.7	0.000076	0.304
572	Beef	25	14.0	0.000187	0.748
573	Beef	25	12.0	0.000160	0.640
574	Beef	25	17.1	0.000228	0.912
575	Hog	25	5.2	0.000069	0.276
576	Hog	25	2.5	0.000031	0.124
577	Hog	25	5.6	0.000075	0.300
578	Sheep	25	6.9	0.000092	0.368
579	Sheep	25	4.3	0.000057	0.228
580	Sheep	25	6.2	0.000083	0.332

to the third decimal place. The method will no doubt be found of particular value in the examination of samples of known origin, especially those of which only very small amounts are available and in cases where the percentage of contained epinephrine is exceptionally minute.

Of the samples upon which results are reported in table I, the first eight are those for which data are given in our previous paper.⁵ It will be noted that the phosphotungstic acid reagent has in general given slightly higher results than the iodate reagent, but still not as high values as obtained by the physiological assay. There is one important exception, however, in the case of sample No. 370 which the phosphotungstic acid reagent shows to be two

⁵ *Loc. cit.*

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and one-half times as active as indicated by its action on the blood pressure. The comparative results reported by Folin, Cannon and Denis upon their samples show, on the other hand, a remarkable agreement between the phosphotungstic acid reagent and the physiological method of assay. It should be noted, however, that these authors used in all cases the same gland extract for both determinations, whereas in our experiments separate weighed samples of the glands were extracted in the particular manner adopted for each method.

From the standpoint of the analyst desiring a control method for commercial desiccated suprarenal glands, the phosphotungstic acid reagent seems to possess several disadvantages. The fact that uric acid and quite a number of other compounds yield exactly the same shade of blue color as epinephrine, indicates that the reaction is not as specifically characteristic of epinephrine as is desirable. This fact might in some cases afford a reasonable doubt as to whether the result obtained with a given commercial sample was due wholly to the contained epinephrine. Consequently for samples of unknown origin a confirmatory test for the genuineness of the material would be necessary in order that unreserved confidence might be placed in the results obtained by means of the phosphotungstic acid reagent.

Disadvantages of less importance are that the blue color fades quite rapidly, therefore necessitating very prompt comparisons of the standards and unknowns. Also that the standard uric acid solution is stable for only a few days. In the case of several standards made by me on succeeding days and then compared simultaneously with each other, rather irregular differences in the developed colors were noted. It is not certain, however, whether these were due to the differences in rate of deterioration of the solutions or to slight errors in measuring the 1 cc. portion to which the phosphotungstic acid reagent is added for the color development.

Considering the very small aliquot portion of the sample required for supplying enough solution for colorimetric estimation, it would appear that the amount of suprarenal used for the epinephrine determination could be very materially reduced and possibly some steps of the method shortened or eliminated.

Finally, attention may be called to the gradual separation of

a crystalline precipitate which often appears in the colored solutions before the comparisons in the colorimeter have been completed. Only one sample of phosphotungstic acid was, however, available for my experiments and it may be that this more or less disturbing precipitate resulted from an impurity in this sample and would not be observed with others.

Gold chloride reagent. In the paper of Gautier⁶ it is stated that using a 0.33 per cent aqueous gold chloride solution, one drop added to 10 cc. of a 1 : 500,000 solution of epinephrine gives a rose-violet coloration without warming; two drops added to a 1 : 100,000 solution yield a violet coloration and 30 drops added to a 1 : 10000 solution give a beautiful red color. The significance of these observations was not apparent to me until my experiments with a pure epinephrine solution showed that the maximum color production requires that a definite ratio be maintained between the amount of gold chloride and epinephrine present. Experiments on which this conclusion is based were made both by adding increasing amounts of gold chloride solution to given amounts of 1 : 100,000 epinephrine solution and also the same amount of gold chloride reagent to varying dilutions of epinephrine. Thus:

1:100,000 EPINEPHRINE	H ₂ O	GOLD CHLORIDE REAGENT		APPEARANCE OF SOLUTION
		Per cent conc.	cc. used	
cc.	cc.			
10	0	0.033	0.1	Very faint pink.
10	0	0.033	0.5	Pinkish color.
10	0	0.033	1.0	More intense pink color than preceding; bluish-pink by transmitted light.
10	0	0.33	0.5	Cloudy opalescence by reflected, bluish by transmitted light.
10	0	0.33	1.0	Cloudy opalescence, brick-red by reflected, blue by transmitted light.
5	0	0.33	5.0	Yellow of the gold chloride practically un- changed.
1	9	0.033	1.0	Trace bluish-pink.
2	8	0.033	1.0	Slightly more color than preceding.
5	5	0.033	1.0	Much more intense than preceding.
10	0	0.033	1.0	About twice as intense as preceding.

⁶ *Loc. cit.*

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It is, therefore, seen that if too little gold chloride is used the full color is not produced and if too much is added the solution takes on an opalescent appearance and is characterized by being a pinkish, reddish or yellowish tint by reflected and a bluish color by transmitted light. With a very large excess of gold chloride no epinephrine color is developed at all. The following experiment was made to ascertain the exact ratio of reagent required for the maximum production of color:

EPINEPHRINE SOLUTION 1:100,000	H ₂ O	0.033 PER CENT GOLD CHLORIDE SOLUTION	RELATIVE INTENSITIES OF PINK COLOR PRODUCED
cc.	cc.	cc.	
1.0	8.5	0.5	1
1.0	8	1.0	2
1.0	7	2.0	5
1.0	6	3.0	5 (slightly bluish tinge)
1.0	4	5.0	4 (bluish)

A number of experiments were made with other concentrations of epinephrine and it was found that the maximum color was always produced with approximately the same ratio of gold chloride as found in the above experiment. The actual amount of epinephrine present was known since the solution had been prepared by weighing out accurately the carefully purified active principle; the amount of gold in the reagent was also determined carefully and it was found that the ratio of epinephrine to gold in the solution of maximum color was: 0.000010 gram epinephrine to 0.000034 gram gold or, calculated to the molecular basis, 1 gram mol. epinephrine to 3.14 gram atoms Au.

The shade of pink color produced with gold chloride matches very closely that developed by means of potassium iodate as previously described.⁷ In regard to the actual intensity of the developed color it was found on comparing the tubes in which the maximum color had been produced with the permanent standards made in connection with the iodate method referred to above, that for a given amount of epinephrine (at least at great dilutions), approximately five times as much color is produced with the gold reagent as with potassium iodate. The observation of

⁷ Hale and Seidell: *Amer. Journ. of Pharm.*, lxxxiii, p. 551, 1911.

Gautier in regard to the exceptional sensibility of the gold chloride reaction is, therefore, confirmed by the present experiments.

From the quantitative standpoint, however, since relatively small differences in the ratio of gold to epinephrine were found to make quite appreciable differences in the intensity of color produced, it is evident that an accurate method based on the use of gold chloride must provide steps to first show the approximate amount of epinephrine present. Although this difficulty might not be insurmountable, it seems inadvisable to make further experiments with this reagent until others, which appear not to be incumbered by such limitations, have been studied.

Manganese dioxide reagent. A consideration of many of the reagents so far proposed for the colorimetric detection of epinephrine shows that they are, for the most part, characterized by their relatively mild oxidizing power. The pinkish colors produced resemble each other fairly closely, therefore, indicating that a similar reaction is set up in each case. As has been pointed out for the potassium iodate reaction, the pink color is more or less modified by the yellowish extractive material derived from the sample during the boiling required to develop the pink epinephrine color.⁸ The intensity of the pink is not sufficient to overcome the effect of the yellow and therefore disturbing variations occur with different samples. In the case of the gold chloride reagent, although a greater intensity of epinephrine color is obtained and this might be sufficient to offset the extractive colors, the necessity for controlling the relative amount of reagent prevents its satisfactory use. It would, therefore, appear that a reagent is needed which can be used in reasonably variable excess

⁸ An experiment was made which confirms the assumption that it is the required boiling which so seriously modifies the shade of the developed color. Thus, two 0.010 gram portions of each of four samples of glands were mixed with 10 cc. volumes of water, one set heated to boiling for a few minutes and the other not; the eight mixtures were filtered after two hours. Those which had been heated showed, tube for tube, an unmistakably more brownish appearance than those which had not been heated. Although there was a just perceptible difference in the amount of coloring matter in the unheated tubes, very marked differences were apparent between the four heated tubes, thus showing how two samples with equivalent amounts of epinephrine could easily yield quite different shades of the pink epinephrine color.

and will develop the epinephrine color without the aid of heat. Of those producing the characteristic pink color which have so far been brought to my attention none appear to fulfil the above-named requirements. In looking about for such a reagent I tried among others, solid manganese dioxide and found that by simply shaking together in a test tube water and a small pinch each of desiccated suprarenal glands and powdered manganese dioxide that the characteristic pinkish epinephrine color slowly develops without the aid of either acid or heat. The maximum intensity appeared to be reached in less than an hour and in common with the pink colors developed by means of mild oxidizing agents, fading proceeded at an exceedingly slow rate. A number of difficulties were of course encountered in adapting this reagent to the satisfactory quantitative determination of epinephrine in desiccated suprarenal glands and the succeeding steps in the development of the method will, therefore, be described in detail.

The first point to be decided, was the optimum amount of manganese dioxide to use. Experiments were made both with pure epinephrine solution and gland samples, and it was found that for 10 cc. of the former containing approximately 1 part per 200,000, 0.0005 gram of MnO_2 developed slightly less color than 0.001 gram, and that 0.010 gram gave no more color than 0.001 gram. In the case of the desiccated glands it was found that for 0.005 gram of sample plus 10 cc. of water, appreciably less color was obtained with 0.0005 than with 0.002 gram of MnO_2 , but that 0.01 gram did not increase the color. In both cases it was found that more than 0.10 gram of MnO_2 diminished the amount of color produced. From these experiments it was concluded that, for 10 cc. portions of epinephrine or gland solution containing up to 1 part per 50,000, 0.005 gram of MnO_2 would develop the maximum color.

The question of the effect of the presence of hydrochloric acid was studied with results showing that excess of free acid causes a slight diminution of color. The difference caused by 0.1 to 1.0 cc. of 0.1 N HCl per 10 cc. of solution, containing approximately 1 : 300,000 of epinephrine, is, however, barely perceptible, but more acid causes a distinct diminution in color.

The time required for maximum development of color was found to be about one-half hour, but in order to be on the safe side one

hour was always allowed before filtration of the solution preparatory to comparison against the standards. After filtration a just perceptible fading occurs after twenty-four hours' standing.

As in the case of the potassium iodate method annoying differences in the shades of color produced by the reagent in pure epinephrine solutions and the aqueous extracts of the gland samples were observed. This, of course, prevented satisfactory comparisons between the epinephrine standards and the gland samples and left as the only improvement of the new method over the old, the elimination of the heating step and the addition of a definite amount of acid. It was recognized that some way of overcoming this difficulty would have to be found. The first experiments were directed toward procedures for modifying or reducing the amount of foreign color in the gland solutions. These, however, led to negative results and will only be mentioned briefly. Thus, by filtering out the gland sample before adding the reagent, slightly cloudy solutions as dissimilar from the epinephrine standards as those from which the sample was not previously filtered, were obtained. Efforts to develop the color in much more concentrated solutions and dilute to the proper color intensity showed that no advantage was to be gained in this way. The amount of color produced appeared to be independent of the concentration of the active principle at the period of the development of the color.

Since it did not appear feasible to modify the tint of color yielded by the gland samples to make it conform to that obtained from pure epinephrine, it was decided to attempt the reverse procedure, namely, to modify the tint obtained from epinephrine to correspond with that yielded by the desiccated glands. The obvious way to do this is of course to add epinephrine-free gland powder to the solution of the pure active principle, and develop the color exactly as is done with glands of ordinary activity. Preliminary experiments showed this plan to be entirely feasible and a series of color standards were therefore made as follows from epinephrine solution (prepared from the purified principle) and suprarenal sample No. 370, which previous physiological experiments and determinations by the iodate method had shown to be practically free of epinephrine.

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TUBE NO.	1:100,000 EPINEPHRINE SOLUTION	H ₂ O	SAMPLE NO. 370	MnO ₂	TIME BEFORE FILTRATION	GRAM EPINEPHRINE PER 10 CC. EQUIVALENT TO COLOR INTENSITY OBTAINED
	cc.	cc.	gram	gram	hour	
a	1.0	9.0	0.010	0.005	1	0.00001
b	2.0	8.0	0.010	0.005	1	0.00002
c	3.0	7.0	0.010	0.005	1	0.00003
d	4.0	6.0	0.010	0.005	1	0.00004
e	6.0	4.0	0.010	0.005	1	0.00006
f	8.0	2.0	0.010	0.005	1	0.00008
g	10.0	0.0	0.010	0.005	1	0.00010

On comparing the tint of color obtained by adding the MnO₂ reagent to various suprarenal samples with the tubes of the above scale the agreement between the shade of color developed in the samples and in the standards was all that could be desired. No difficulty whatever was experienced in locating exactly the position of the sample tube in the scale of standard tubes. The determinations made upon the set of suprarenal samples by this method are given in table II. In each determination 0.01 gram of sample, 0.005 gram of MnO₂ and 10 cc. of water were taken, and the mixture filtered into a test tube at the end of one hour.

It may be mentioned here that for all the experiments described in this paper, except those upon the phosphotungstic acid method, test tubes of diameter and length of approximately 2×17 cm. were used. The 10 cc. of solution chosen for all determinations gave a depth of about 4 cm. and the comparative intensities of colors were estimated by observing the tubes from the side, holding them in pairs or threes over a white sheet of paper. Of course any convenient size of test tube may be chosen, but it is important that only those of approximately the same diameter be used in a given series of determinations.

The results obtained by this method are seen to be, with the exception of several of the samples of low activity, higher than found by means of the phosphotungstic acid reagent. In the case of the first eight samples they approach fairly closely the results obtained by the physiological assay as reported in our previous paper. It cannot, therefore, well be claimed that the failure to use hydrochloric acid and heat has resulted in incomplete extrac-

TABLE II.

Percentage of epinephrine in commercial desiccated suprarenal glands as determined by the manganese dioxide method.

SAMPLE NO.	SOURCE	DEVELOPED COLOR = THAT OF STANDARD TUBE NO.	CALC. PER CENT EPINEPHRINE
362	Sheep	f	0.8
363	Sheep	f	0.8
365	Sheep	g	1.0
366	Sheep	b	0.2
367	Sheep	f	0.8
368	Sheep	b-c	0.25
369	Sheep	d	0.4
370	Sheep	less than a	0.0
416	Sheep	d	0.4
417	Beef	diluted $\frac{1}{2}$ = f	1.6
418	Hog	c-d	0.35
572	Beef	diluted $\frac{1}{2}$ = e	1.2
573	Beef	f	0.8
574	Beef	diluted $\frac{1}{2}$ = e-f	1.4
575	Hog	c	0.3
576	Hog	a	0.1
577	Hog	c-d	0.35
578	Sheep	d-e	0.5
579	Sheep	a-b	0.15
580	Sheep	d-e	0.5

The order of decreasing intensity of color in the samples as determined by comparison with each other was as follows, the per cent found above by comparison with standards being given in parentheses.

365	(1.0)	$\frac{1}{2}$ (572)	(0.6)	368	(0.25)
362	(0.8)	578	(0.5)	575	(0.3)
363		580		366	(0.2)
373		369		579	(0.15)
367	$\frac{1}{2}$ (417)	416	(0.4)	576	(0.1)
$\frac{1}{2}$ (574)		577		370	(0.0)
		418			
	(0.7)		(0.35)		

tion of the epinephrine from the desiccated glands. This appears of particular interest since it is usually considered necessary to digest with dilute acid in order to obtain the full activity of the glands. It, therefore, appeared of interest to test the reaction of aqueous extracts of the glands. This was done by violently shaking a few tenths of a gram of each of several samples in a test

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tube with a few cubic centimeters of water and immediately adding blue litmus paper. A distinct red color was obtained in all cases.

There is one point in connection with the proposed new method which was at first thought to limit its general use, and that is the required epinephrine-free desiccated suprarenals for preparing the standards. It is possible that such material is not of frequent occurrence and a substitute or at least a method for its preparation appears necessary. A simple means for destroying the contained epinephrine in a given sample can no doubt be found, but an experiment made with desiccated thyroid glands showed that this product could readily be substituted for the inactive suprarenals and methods for destroying the active principle in suprarenal glands were, therefore, not sought. The shade and intensities of the developed color corresponded closely with the standards described above and it is evident that perfectly satisfactory results can be obtained with standards made from pure epinephrine solutions and desiccated thyroid gland.

A more serious handicap, however, to the present method, as so far described, is that the costly and difficultly purified epinephrine must be available as the ultimate basis of the determinations. It was, therefore, recognized as necessary that artificial color standards which can readily be reproduced by different analysts must be devised. Experiments along this line were, therefore, next undertaken.

Artificial permanent color standards for the manganese dioxide method. As in the case of the color developed with potassium iodate that obtained by means of manganese dioxide is also a mixture of red and yellow. As mentioned in the previous paper permanent standards composed of mixtures of aqueous solutions of cobalt chloride and of potassium platonic chloride were proposed. In the present case it was found that gold chloride could be substituted for the platinum solution and since a standardized solution was on hand, mixtures of it and cobalt chloride solution were used to match the color developed from pure epinephrine and aqueous extracts of the gland samples.

The concentrations of the cobalt and gold solutions which appeared most convenient were: 2 grams crystalline cobalt chloride, $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, dissolved in water, 1 cc. of concentrated HCl added and the mixture diluted to 100 cc. The approximately 0.3 per

cent gold chloride solution used for the previously described experiments was analyzed by evaporating 40 cc., to which a small amount of sugar was added, to dryness, igniting, and weighing the gold. On the basis of this determination the solution was diluted with water to contain exactly 0.1 gram of Au per 100 cc.

By measuring these two solutions from burettes it was found that a mixture of 10 cc. of the cobalt solution and 3 cc. of the gold solution matched exactly the tint of color developed from 10 cc. of a 1 : 100,000 pure epinephrine solution by means of 0.005 gram manganese dioxide as already described. A series of readings in the Duboscq colorimeter showed that a depth of 25 units of the epinephrine color was of exactly the same intensity as a depth of 20 units of the cobalt plus gold color. Therefore to prepare an artificial standard for estimating the epinephrine in pure aqueous solutions, 10 cc. of cobalt solution + 3 cc. of gold solution + 3.25 cc. H_2O gives a color of exactly the tint and intensity as that yielded by 1 : 100,000 epinephrine solution.

As has already been pointed out the tint of color obtained from gland samples does not match exactly that obtained from the pure epinephrine, it was, therefore, necessary to prepare a series of artificial color standards which would match exactly the colors of the standard scale prepared as already described from pure epinephrine and inactive desiccated suprarenal gland. It was at first thought that since the differences in tint between the color obtained from the glands and pure epinephrine are due only to the yellowish extractive material obtained from the gland itself, that the simple addition of an amount of gold solution equivalent to this extraneous color, to dilutions of the mixture found above to be equal to the color from 1 : 100,000 epinephrine, would give the desired scale of colors. This proved not to be the case, however, and the reason appears to be that, although each sample of glands may yield nearly the same amount of yellow coloring matter, the amount of pink plus yellow epinephrine color varies and consequently there are obtained mixtures of varying ratios of pink and yellow which do not correspond to the color scale made as outlined above.

It therefore became necessary to determine empirically the amounts of cobalt solution, gold solution and water which would

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exactly match each tube of the standard scale prepared from epinephrine plus inactive gland. This was not a very tedious procedure, however, and no difficulty was experienced in obtaining artificial color standards which could not be distinguished from those of the epinephrine. In order to eliminate individual variations to as great an extent as possible the empirically determined proportions of the cobalt, gold and water were plotted on cross-section paper and from the curves so obtained the following table was constructed.

TUBE NO.	COBALT SOLUTION	GOLD SOLUTION	H ₂ O	GRAM EPINEPHRINE + 10 cc. H ₂ O + 0.010 gm. INACTIVE SUPRARENAL + 0.003 gm. MnO ₂ TO PRODUCE EQUIVALENT COLOR
	cc.	cc.	cc.	
1	1.15	0.70	8.15	0.00001
2	1.85	0.95	7.20	0.00002
3	2.40	1.10	6.50	0.00003
4	2.95	1.25	5.80	0.00004
5	3.50	1.30	5.20	0.00005
6	4.05	1.35	4.60	0.00006
8	5.15	1.55	3.30	0.00008
10	6.30	1.75	1.95	0.00010

The 10 cc. mixtures made according to this table were sealed in carefully selected test tubes and form the permanent standards for the estimation of epinephrine in desiccated suprarenal glands. On the basis of 0.01 gram portions of sample, 0.005 gram MnO₂ and 10 cc. of water the developed colors as compared with the above tubes correspond to 0.1 per cent epinephrine in the case of tube No. 1 and 1 per cent in the case of tube No. 10. Therefore, in estimating the epinephrine in a given sample it is only necessary to weigh out the 0.01 gram, add the MnO₂ and water, shake thoroughly and allow to stand one hour, filter into a test tube of dimensions corresponding as nearly as possible to those of the tubes of the above scale and ascertain by comparison against a white background, with which of the standard tubes the sample color corresponds.

In cases of samples which contain more than 1 per cent of epinephrine the filtered solution may be diluted one-half and read against the scale. Under such conditions, however, the tints

may not correspond exactly since by this procedure only one-half of the yellow coloring matter of the sample is present. The difference in tint is usually, however, insufficient to appreciably affect the reading. For very accurate work, therefore, it is better, with samples of high epinephrine content, to take a proportionally smaller amount than 0.01 gram and make up the difference with epinephrine-free gland powder before adding the MnO_2 and water for development of the color.

It should be mentioned that the artificial color standards as above described have been repeatedly checked against known amounts of the purified ash-free epinephrine and furthermore determinations have been made upon the twenty samples of commercial desiccated suprarenal glands enumerated in table II, using the artificial standards for comparison with results agreeing satisfactorily with those there shown.

The question as to whether various samples of manganese dioxide would yield equal intensities of color was considered and an experiment was made in which five different samples of the reagent were used. Satisfactory agreement in the intensities of the colors was obtained in all cases. In this connection, however, it should be mentioned that while making some preliminary experiments with the method at my request, Mr. Frederic Fenger of Chicago, Ill. found that certain samples of manganese dioxide labeled as being chemically pure gave low results. So far as I know the manganese dioxide used by me was of the grade sold as technically pure. The label on one of the samples indicated that the material was granular pyrolusite. The exceptional results obtained by Mr. Fenger indicate that possibly certain samples designated as very pure manganese dioxide are not as suitable reagents for the present method as the unpurified product or the finely powdered mineral pyrolusite.

A comparison of the results obtained with the phosphotungstic acid method and with the manganese dioxide method shows that the latter gives, in practically all cases except with the samples of low activity, higher results than the former. In fact, if the results by the manganese dioxide method are assumed to be correct, then the phosphotungstic acid method apparently gives low results with the high samples and high results with the samples of low epinephrine content. The question as to which method

will eventually be found to give results approaching nearest those found by physiological assay cannot be predicted at present.

SUMMARY.

Attention is called to the defects of the previously proposed potassium iodate method for commercial suprarenal glands and explanations of the sources of error in it are given.

The phosphotungstic acid method of Folin and Denis has been applied to a series of desiccated suprarenals and, although it possesses many excellent features, certain disadvantages which apparently render it unsuitable for the standardization of commercial glands, are pointed out.

The gold chloride reagent was found to yield a maximum color only when a definite ratio was maintained between the amount of epinephrine and of gold, thus seriously restricting the practical quantitative application of this reagent.

The new method proposed in the present paper consists in the use of manganese dioxide as the reagent for developing a color with aqueous epinephrine solutions or suprarenal gland extracts. The color so developed is estimated by comparison with artificial color standards made by mixing cobalt chloride, gold chloride and water.

THE NATURE OF THE DEPRESSOR SUBSTANCE OF THE DOG'S URINE AND TISSUES.

BY ALONZO ENGLEBERT TAYLOR AND RICHARD M. PEARCE.

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The normal urine,¹ the kidney² and the pancreas³ of the dog have been shown by one of us to contain a depressor substance, the action of which can be strikingly demonstrated in kymographic tracing taken after intravenous injection. A number of investigators have studied the effect of the depressor organ extracts from the physiological point of view, and have attempted to bring the characteristic action of these extracts into relation with peptone intoxication and anaphylactic shock, but without success. Recently, Whipple⁴ and his associates have shown that the contents of an obstructed duodenal loop (dog) contain a powerful depressor substance very similar in action to that of the urine and kidney of the dog.⁵ This observation of Whipple, in view of the studies of Dale and Laidlaw⁶ of the effects on the blood pressure of β -iminazolyethylamine produced from histidine when carbon dioxide is split off (Ackermann),⁷ which effect is similar in every way to that of the urine and organ extracts of the dog, and again the

¹ Pearce: *Journ. of Exp. Med.*, xii, p. 128, 1910.

² Pearce: *Ibid.*, xi, p. 430, 1909.

³ Eisenbrey and Pearee: *Proc. Soc. of Exp. Biol. and Med.*, viii, p. 93, 1911.

⁴ Whipple, Stone and Bernheim: *Journ. of Exp. Med.*, xvii, pp. 286, 307, 1913.

⁵ Compare Pearee and Eisenbrey: *Amer. Journ. of Physiol.*, xxvi, p. 26, 1910.

⁶ Dale and Laidlaw: *Journ. of Physiol.*, xli, p. 318, 1910; xliii, p. 182, 1911.

⁷ Ackermann: *Zeitschr. f. physiol. Chem.*, lxx, p. 504, 1910.

work of Mellanby and Twort⁸ and Berthelot and Bertrand⁹ on the isolation from the intestine of a bacillus capable of producing β -iminazolyethylamine from histidine, suggested that the substance responsible for the fall of blood pressure in peptone intoxication and anaphylactic shock¹⁰ and following the injection of urine and organ extracts (Popielski's "vasodilatin") and Whipple's duodenal loop contents might be one and the same substance. With this possibility in view attempts were made to isolate it or the substance to which it is attached, from dog's urine, pancreas and duodenal loops by the methods of Kutscher and Lohmann and of Engeland. Our experiences have been uniformly negative, and since the results indicate the definite inadaptability of our present methods for the chemical isolation of such substances, presumably bases, we feel that record should be made of our negative results.

Our findings in brief are as follows, being the results of work with the collected urines of three dogs, the collected fluids of three isolated loops of small intestine and of the autolyzed pancreas of ten dogs, toluol or chloroform or both being employed as preservative in all cases. In each case the presence in the original fluid of a strongly depressor substance was first determined by kymographic tracings, following intravenous introduction. Whenever such solutions are carefully precipitated by phosphotungstic acid and hydrochloric or sulphuric acid, with great care in avoidance of excess of reagents, especially of acids, the depressor action remains in the precipitate, and may be recovered therefrom after removal of the phosphotungstic acid. If the precipitation be done however with excess of acid, as commonly advised, the depressor action is destroyed, and is not to be recovered from precipitate or filtrate. When isolation is attempted by means of silver, as in the method of Kutscher and Lohmann, the depressor action is invariably lost; the substance causing it is not to be recovered from precipitate or filtrate. When the solution is submitted to the method of Engeland, the depressor action is likewise lost, as before.

⁸ Mellanby and Twort: *Journ. of Physiol.*, xlv, p. 53, 1912.

⁹ Berthelot and Bertrand: *Compt. rend. de l'Acad. des Sci.*, cliv, p. 1643, 1912.

¹⁰ Compare Modrakowski: *Arch. f. exp. Path. u. Pharm.*, lxix, p. 67, 1912.

Working with the extract of autolyzed pancreas, we have been able to show that the depressor substance persists after complete removal of proteins (above and including peptone) by tannic acid, *i.e.*, the depressor action remains in the final filtrate. It is therefore not related to the peptone or other protein. On attempting to isolate the substance carrying the activity from this protein-free solution by the method of Kutscher and Lohmann it was lost, as before.

The substance carrying the action is not precipitated by silver nitrate in the absence of barium hydrate; precipitated in the presence of barium hydrate, it is destroyed. Obviously, slight excesses of either acid or alkali operate to destroy, *i.e.*, to set up reactions whereby the chemical nature of the hypothetical substance is so altered as to deprive it of depressor or other toxic properties.

β -Iminazolyethylamine (the result of bacterial action or in the commercial form, Ergamine) and dimethyl guanidine pass unscathed through the phosphotungstic acid and silver methods of precipitation; they are therefore not concerned with the depressor substances under investigation. Recently Heyde and Vogt¹¹ have reported experiments tending to suggest that methyl guanidine is the toxic substance in the urine following extensive superficial burns. From our extract of autolyzed pancreas, no methyl guanidine could be isolated.

Throughout this investigation, at certain stages, small fractions of precipitate and filtrate were removed for blood pressure observations. As at times the disappearance of the depressor substance coincided with the advent of a pressor substance, a few observations on this phase of the work may not be out of place.

The fluid from the mucosa and contents of duodenal loop after heating at 60°C. for an hour and the filtrate after boiling caused a fall of pressure, but the bases finally extracted caused a slight rise. The extract of the pancreas after heating gave a decided fall, but the purified bases, a rise in pressure.

On the other hand, the preparations from the urine, both filtrate and precipitate after phosphotungstic acid, carefully purified, were inert.

Control observation with Ergamine (the commercial name under

¹¹ Heyde and Vogt: *Zeitschr. f. d. ges. exp. Med.*, i, p. 59, 1913.

which β -iminazolylethylamine is put out by the Burroughs, Wellcome Company) given intravenously show that the fall in pressure due to urine and pancreas and duodenal loop contents is essentially similar. As, however, β -iminazolylethylamine can be recovered by the chemical methods we have used and the depressor substance of animal tissue and fluids cannot, the two cannot be identical.

SUMMARY.

Attempts to isolate by the methods of Kutscher, Lohmann and Engeland the depressor substance occurring in the urine, pancreas and duodenal loops of the dog, and which have an effect on blood pressure analogous to that of β -iminazolylethylamine, have failed.

ON THE DERIVATION OF ETHYL ALCOHOL CONTAINED IN THE MUSCLE.

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Pennsylvania.)*

(Received for publication, June 6, 1913.)

Freshly killed muscle yields on distillation traces of ethyl alcohol. This alcohol may have been derived from bacterial processes within the alimentary tract. To exclude this source, it is not enough to starve the animal prior to its death. The proteins contained in the alimentary juices, the secretion of which continues to a greater or less extent during starvation, undergo cleavage; from the amino-acids sugar may be formed and this sugar then fermented to alcohol. The only correct method of exclusion of the bacterial processes within the alimentary tract consists in complete removal of the tract. The following report contains the details of such an experiment; and the result indicates that traces of ethyl alcohol are contained in freshly killed muscle under circumstances that practically exclude the alimentary tract as the site of formation.

The operation of complete removal of the alimentary tract in the dog is a difficult operation, which has been skilfully and ingeniously accomplished for me by Dr. Max M. Peet, who will elsewhere report upon the operative features of the procedure. For the purpose of the experiment in hand, it was necessary that no anesthetic be employed that contained or could yield any alcohol, aldehyde or ketone groups; that the circulation of the pancreas and the integrity of this gland be maintained; and that the animal should survive the operation long enough to permit of the combustion of any traces of alcohol that might have been absorbed from the intestine prior to the operation. The anesthetic employed was nitrous oxide with oxygen, and the anesthesia, which was very complete, was in every way satisfactory except that

hemorrhage was free. The entire tract was removed, including the lower end of the oesophagus and the rectum. The animal recovered from the shock of the operation, saline infusion having been used to offset the loss by hemorrhage. Urine was drawn by catheter about eight hours after the operation; it contained neither albumin nor sugar, and contained all the usual components, including some indican and ethereal sulphates, presumably absorbed prior to the operation. The dog was killed eighteen hours after the operation, since death was foreseen to be inevitable within a short time, and I wished to permit no opportunity for post-mortem changes to set in. The animal was hurriedly dissected and a little over 3 kilos of muscle were placed in two large distillation flasks with three times the weight of distilled water, and subjected to distillation. Particular attention was paid to the cooling of the condensers, which were very long and effective, and the receiving flasks were surrounded by ice and fitted with traps. The distillations were continued until two-thirds of the fluid was distilled over. The distillate was redistilled in the same manner, and the distillations repeated until the final distillate was reduced to 25 cc. In one distillation the solution was acidulated with hydrochloric acid. The final distillate gave the iodoform reaction, and without wasting the material on a series of more or less indefinite tests, the entire solution was subjected to Buchner's¹ test, the formation of the ethyl ester of *p*-nitrobenzoic acid, by heating with *p*-nitrobenzoyl chloride. A goodly crystallization followed, the collected crystals (weighing 0.3 gram) were twice recrystallized as suggested by Buchner, and the final yield identified by the melting point and the content of nitrogen. The crystals melted at 57.4°. The known melting point of the crystals of this ester is 57°. Two analyses of 100 mgm. of the ester yielded 7.02 and 7.10 mgm. of nitrogen respectively; the calculated percentage is 7.18.

Have we the right to assume that any traces of ethyl alcohol that were contained in the muscle of a starving dog as the result of resorption from the intestine would have been completely burned within eighteen hours, and that the traces found in this experiment may therefore be regarded as derived from the metab-

¹ Buchner: *Berichte*, xxxviii, I, p. 624, 1905.

olism? When one recalls the velocity of oxidation of alcohol it seems fairly certain that the alcohol found could not have been derived from the intestine. The dog, while starving for one day prior to the operation, was in good nutrition; and samples of muscle tested for glycogen gave good reactions.

From what source could this ethyl alcohol have been derived? It seems most natural to relate it to the metabolism of carbohydrate and to infer that it has been derived from lactic acid, just as in yeast fermentation lactic acid is split into ethyl alcohol and carbon dioxide. If this be correct, one further step in the reactions of combustion of glucose will have been made experimentally clear. Years ago Nef and Buchner suggested that methyl glyoxal might be an intermediary stage in the conversion of glucose into lactic acid. This suggestion has been experimentally verified in recent investigations of Dakin.² In a general sense, as once suggested by Bach, the train of reactions involved in the oxidation of glucose might run something like the following, the methyl glyoxal being inserted: glucose \rightarrow methyl glyoxal \rightarrow lactic acid \rightarrow ethyl alcohol \rightarrow acetic acid \rightarrow formic acid \rightarrow carbon dioxide and water, the end products being set free at several stages. Possibly methane might be a stage between acetic acid and formic acid. Dakin³ has recently shown definitely that formic acid is a normal constituent of urine. The whole scheme, while in part entirely hypothetical and in some respects outside the line of usual interpretation, is harmonious with many known facts in the metabolism of plants and bacteria.

It is obvious that animals deprived of the entire alimentary tract offer opportunities for research in many directions that promise to yield information of decisive value in the determination of different problems. From our present experience with two dogs, we believe that, done with expedition, without extreme shock and hemorrhage, these animals would live for days. In a certain sense, such a dog, with a biliary fistula, would be in much the same position as the animal in complete starvation, except that the organism would be free of the products of bacterial life and of bacterial activity upon the alimentary juices. In such an animal,

² Dakin: this *Journal*, xiv, pp. 155, 423, 555, 1913.

³ Dakin: *ibid.*, xiv, p. 341, 1913.

for example, one could determine whether there be an endogenous, metabolic fraction of aromatic bodies—phenol, indol and benzoic acid. With the exclusion of the bile, one could investigate the nature of the metabolic pigment of the urine. One could determine whether the ferments of the blood and urine are derived from the alimentary tract. We hope at no distant date to report upon such and other investigations.

ON THE NATURE OF THE SUGARS FOUND IN THE TUBERS OF ARROWHEAD.

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(Received for publication, June 6, 1913.)

The present paper embodies the result of our study on the nature of the sugars found in the arrowhead tubers (*Sagittaria sagittifolia forma sinensis*), and forms a part of the investigations on the sugars contained in the underground reserve organs of plants, now being conducted in our laboratory.

The arrowhead tuber or Kuwai was selected as the first material to be studied. A search of the literature on *Sagittaria sagittifolia forma sinensis* failed to show the result of special investigations on the composition of its tubers, beyond a brief article by Kellner¹ on their general composition. Consequently, at the beginning of this investigation we have undertaken to test the carbohydrates of the arrowhead tubers and obtained the following result:

	per cent
Water.....	78.16
<i>In water-free substance.</i>	
Starch.....	55.32
Dextrin.....	1.75
Reducing sugars.....	0.67 (as glucose)
Non-reducing sugars.....	5.54 (as sucrose)
Mucic acid producing substance by oxidation....	1.43 (as galactose)
Insoluble in 95 per cent alcohol.....	0.69 (as galactose)
Soluble in 95 per cent alcohol.....	0.74 (as galactose)
Pentosane (including methyl pentosane).....	1.83

As has been shown in the above table, of the carbohydrate constituents, starch is a prominent substance, its amount attaining 55.32 per cent of the dry matter. Sugars are also present in no

¹ König: *Chemie der menschlichen Nahrungs- u. Genussmittel*, Berlin, i, p. 705, 1903.

slight quantity, reaching the amount of 6.21 per cent of the dry matter, and they form an important part of the carbohydrate constituents.

To determine the exact nature of the sugars of the arrowhead tubers, the following investigation was undertaken.

1. Preparation of the syrup.

The arrowhead tubers were peeled of their rind and finely chopped. The chopped parts were allowed to dry in the air, requiring about two weeks to dry them to such a state that they could be ground easily and finely pulverized.

The preparation of the syrup was begun by extracting 100 grams of the finely pulverized material in a Soxhlet apparatus with ether. The residue so obtained, which was freed from oil, after evaporating the ether was placed in a 750 cc. flask fitted with inverted condenser and treated daily with 300 cc. of 95 per cent alcohol heated in a boiling water bath. The extract was at first of a deep yellow color, but it gradually became lighter from day to day. It had a sweetish taste and was nearly neutral to litmus during the entire time of extraction. One week was required to remove the last traces of sugars. The combined extracts were filtered to remove the sediment which was formed on standing and the filtrate was evaporated to a small volume in a partial vacuum. The concentrated liquor was again extracted many times with a small quantity of absolute alcohol, until no more residue was left after the evaporation of the alcohol. The residue (I) so obtained was preserved for later investigation. The extracts were concentrated to a syrupy condition in a partial vacuum and allowed to dry over sulphuric acid. The preparation was repeated about ten times to get a sufficient quantity for investigation.

2. Experiments with the syrup.

A. Qualitative tests. The syrup obtained above, gave the following qualitative reactions:

1. It had a very sweet taste.

2. It reduced Fehling's solution weakly; after inversion with hydrochloric acid, the reducing power was very much enhanced, showing that the non-reducing sugars were present in abundance.

3. Molisch-Udransky's reaction was positive.
4. It gave a characteristic blood-red color by heating with picric acid and a few drops of caustic soda solution (reaction of Braun for glucose).
5. It gave Seliwanoff's reaction very distinctly.
6. It gave Pinoff's reaction for free fructose with ammonium molybdate and acetic acid.
7. It gave characteristic red color by heating in a boiling water bath for exactly one minute with resorcin and alcohol-sulphuric acid mixture according to Pinoff.
8. It did not show any pentose reaction by the phloroglucin method.
9. Mucic acid was produced upon oxidation with nitric acid of 1.15 sp. gr., in the usual manner.
10. Saccharic acid was detected as acid potassium salt in the oxidized solution separated from the crystals of mucic acid by the usual method.
11. It rotated the plane of polarization toward the right; after inversion it was almost inactive.
12. It produced no characteristic mannose phenyl hydrazone with phenyl hydrazine. When the mixture was warmed in a boiling water bath with acetic acid, a yellowish crystalline osazone was produced. Even after inversion, mannose phenyl hydrazone was not detected.
13. Two drops of the syrup were placed on an object glass and were seeded respectively with a crystal of glucose, fructose, galactose, maltose, sucrose and raffinose. After twenty-four hours, the drop which had been seeded with sucrose showed the formation of new crystals, while the others remained unchanged.

From the above qualitative reactions it is safe to conclude that the syrup contains both reducing and non-reducing sugars and that the presence of glucose, fructose, galactose and sucrose is highly probable. Moreover, it is probable that fructose in a free form is present, because the reaction 6, according to Pinoff, is only produced by free fructose while other sugars which contain the fructose molecule in combination as sucrose do not show the same color reaction.

B. Isolation of sucrose. When the syrup was left untouched nearly twenty-four hours, it was found thickly laden with fine crystals. A small amount of 95 per cent alcohol was added to the syrup, mixed, filtered by suction, and washed with absolute alcohol and ether. The sugar thus obtained was slightly yellowish in color, but upon recrystallization from alcohol it became perfectly white and left no ash on ignition. After drying over sulphuric acid in a vacuum, its melting point was determined and found to be 159°C.

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0.5 gram of the dried sugar was dissolved in water and made up into 25 cc., and polarized in 200 mm. tube in a Schmidt and Haensch half shadow polariscope. The rotation was found to be 7.7 on the scale toward the right. The specific rotatory power of this sugar is

$$[\alpha]_D = \frac{7.7 \times 0.346 \times 25}{0.5 \times 2} = + 66.6^\circ \text{ (at } 20^\circ \text{)}$$

The melting point and specific rotatory power indicate that the sugar is sucrose.

C. Osazone tests. The mother liquor filtered off from the crystals of sucrose was evaporated again to a syrup. After standing for about two days, a small amount of sucrose crystals was again formed in it. The crystals were removed as in the above experiment, and the filtrate was concentrated once more to a syrup. It did not show any sign of forming new crystals even after one week's standing. An attempt was then made to separate and detect the sugars as osazones.

1. One gram of the syrup, 2 grams of phenyl hydrazine hydrochloride, 3 grams of sodium acetate and 20 cc. of water were mixed and heated in a boiling water bath. After fifteen minutes yellowish crystals were produced. At the end of one hour and a half the crystals were examined under a microscope. No other forms, besides the stellate form of the yellow needle-shaped crystals of phenyl glucosazone, were observed. When cooled, it was filtered and washed with a little water. The yellow crystals thus obtained were recrystallized from 60 per cent alcohol and dried over sulphuric acid in a vacuum. The melting point was determined and found to be 204° , which coincides with that of phenyl glucosazone.

The filtrate from the crystals of phenyl glucosazone was heated and concentrated again in a boiling water bath. This produced a second crop of very fine crystals of brownish-yellow color, and their form was identical with that of phenyl galactosazone prepared from pure galactose in our laboratory. After heating for about an hour, it was filtered and washed with a little water. The crystals were recrystallized and dried over sulphuric acid in a vacuum. The melting point was determined and found to be $193\text{--}194^\circ$. The crystalline form and melting point indicate that the osazone is probably phenyl galactosazone.

2. One gram of the syrup was dissolved in 20 cc. of water and inverted with hydrochloric acid in a boiling water bath for about thirty minutes. After it was neutralized with sodium carbonate, 2 grams of phenyl hydrazine hydrochloride and 3 grams of sodium acetate were added and heated in a boiling water bath, exactly in the same manner as described above. In this case, none of the other osazones besides the considerable quantity of both glucosazone and galactosazone were obtained.

The osazone tests which were made to separate and detect the sugars in the syrup failed to give a more favorable result than that of the qualitative reactions as already mentioned. But, as the result of this experiment, the absence of maltose may be inferred, because maltosazone can easily be distinguished from the glucosazone in its crystalline form, though its melting point is almost similar to that of the latter. The formation of galactosazone from the original syrup in this case is noteworthy, since the presence of free galactose in nature, up to the present time, had not been reported except by Lippmann² who proved its presence in the hoar-frost-like coating of berry ivy after a sudden night frost in autumn. As to whether the formation of galactosazone from the original syrup is due to the presence of free galactose or to a slight inversion of some non-reducing sugar yielding galactose is not yet decided; and the question remains to be solved in the future.

3. Experiments with Residue I.

A. *Qualitative tests.* The residue (I) gave the following qualitative reactions:

1. It had a sweetish taste.
2. It did not reduce the Fehling's solution directly; after inversion with hydrochloric acid, it reduced very strongly showing the presence of non-reducing sugars.
3. It did not give the characteristic reaction of pentose with phloroglucin and hydrochloric acid.
4. Molisch-Udransky's reaction was positive.
5. It did not give the characteristic color reaction of Braun with pieric acid and caustic soda.
6. It gave the characteristic fire-red color of ketose with resorein and hydrochloric acid (Seliwanoff's reaction).

* Lippmann: *Berichte*, xliii, pp. 3611-3612, 1910.

7. It did not give the characteristic color reaction of free fructose with ammonium molybdate and acetic acid.

8. Upon oxidation with nitric acid of 1.15 sp. gr., mucic acid was produced.

9. From the filtrate of the mucic acid crystals, saccharic acid was separated and detected as acid potassium saccharate by the usual method.

10. It did not produce any crystals with phenyl hydrazine hydrochloride and sodium acetate. When the mixture was warmed in a boiling water bath for about thirty minutes, a few crystals of the yellowish glucosazone were produced. After inversion, the glucosazone and galactosazone were formed in abundant quantity by heating for about fifteen minutes.

11. It rotated the plane of polarization toward the right both before and after inversion; though in the latter case the rotation was reduced.

From the above qualitative tests, it is clear that the residue (I) under examination contains some non-reducing sugars which may yield glucose, galactose or fructose.

B. Isolation of sucrose. The residue (I) was dissolved with 95 per cent alcohol and evaporated to a syrup. It did not show any sign of forming crystals of its own accord, even after one week's standing. An attempt was then made to purify the syrup by means of basic lead acetate. The syrup was diluted with a sufficient quantity of water, to which a suitable quantity of basic lead acetate solution was added and the mixture well shaken. The fluid soon became turbid and after standing for a little while, a small amount of flocculent precipitate was formed. After separating the precipitate by filtration, a further quantity of basic lead acetate and ammonia was added to the filtrate, when a large amount of a flocculent white substance was precipitated. The insoluble lead compound was collected on a filter with suction, well washed with water, suspended in water and decomposed by hydrogen sulphide. After decomposition was complete, it was filtered and well washed with water, and then the filtrate was evaporated to a small volume in a partial vacuum. The syrup was next extracted with boiling 95 per cent alcohol and separated into two parts, soluble and insoluble, the former being the larger in quantity. The insoluble part of a slightly dark color was designated as Residue II and preserved for later investigation. The soluble part was again concentrated to a syrup.

The purified syrup did not produce any marked crystals even after standing for about one week. Hence, an attempt was once

more made to clarify the syrup by means of absolute alcohol, i.e., the syrup was extracted many times with a small quantity of absolute alcohol until no more residue was left after evaporation of the alcohol. The insoluble part was reserved (Residue III). The extracts were united and concentrated again to a small bulk.

When the twice purified syrup was left untouched for about twenty-four hours, it was found thickly laden with fine crystals. A small amount of 95 per cent alcohol was then added to the syrup, mixed, filtered with suction and washed with absolute alcohol and ether. The sugar thus obtained was perfectly white in color and left no ash on ignition. It was identified as sucrose by its melting point (160°) and optical rotation ($+66.53^{\circ}$).

4. Experiment with Residue III.

Qualitative tests applied to Residue III showed almost the same reactions as those of the Residue I. A trial was then made to separate and detect the sugar which contains the galactose group. First, we determined how much mucic acid would be produced from the residue by oxidation as follows: 0.2 gram of the residue was put in a small beaker, to which nitric acid of sp. gr. 1.15 was added, and oxidized in a boiling water bath as in the usual manner. The mucic acid produced was collected on the filter and weighed, 0.0065 gram corresponding to 3.25 per cent.

The specific rotatory power of the residue was found to be $+83.35^{\circ}$. If the sugar under question be raffinose, the amount of this sugar corresponding to 0.0065 gram of mucic acid would be 0.087 gram according to Creydt.³ If we assume that sucrose is present besides raffinose, the quantitative ratio of sucrose and raffinose in Residue III would be 1.3:1. Upon this ratio, if we calculate the specific rotatory power of the sample, we will then find the value $+82.98^{\circ}$ which coincides well with that actually observed.

A small amount of methyl alcohol was added to the total residue (III), well mixed and decanted. This operation was repeated until the bulk of the residue insoluble in methyl alcohol was reduced to about half of its original volume. The combined extracts were

³ Creydt: *Zeitschr. d. Ver. d. d. Zuckerind.*, xxxvii, p. 153; Lippmann: *Chemie d. Zuckerarten*, Braunschweig, 1904, ii, p. 1652.

evaporated to a syrup. The syrup did not show any sign of forming crystals even after standing for seven days. Absolute alcohol was then added to the syrup to remove matters soluble in the alcohol, well mixed and decanted. The residue insoluble in absolute alcohol was dried in vacuum over sulphuric acid, and its specific rotatory power was found to be $+92.27^\circ$.

$$[\alpha]_D = \frac{0.4 \times 0.346 \times 10}{0.015 \times 1} = +92.27^\circ \text{ (at } 20^\circ\text{)}$$

The residue was again treated with methyl and absolute alcohol to remove impurities. The substance finally obtained was almost tasteless. The specific rotatory power was determined and found to be -103.8° .

The residue was once more purified in the same manner as before. The specific rotatory power was again determined and found to be constant.

$$[\alpha]_D = \frac{0.3 \times 0.346 \times 10}{0.01 \times 1} = +103.8^\circ \text{ (at } 20^\circ\text{)}$$

Next, we determined the quantity of mucic acid which is produced from the residue by oxidation. 0.0834 gram of the residue and nitric acid of sp. gr. 1.15 were mixed in a small beaker and oxidized in a boiling water bath in the usual way. The mucic acid produced was collected on a filter and weighed, 0.0065 gram corresponding to 7.79 per cent.

According to Tollens,⁴ the specific rotatory power of raffinose is $+103.0^\circ$ or $+104.0^\circ$ while Lippmann⁵ found it to be $+105.0^\circ$ and $+104.95^\circ$. The production of mucic acid from 0.1 and 0.075 gram raffinose according to Creydt⁶ is 0.009 and 0.0056 gram corresponding to 9.0 per cent and 7.5 per cent respectively.

Consequently it is concluded that the residue is probably raffinose.

⁴ Tollens: *Zeitschr. d. Ver. d. d. Zuckerind.*, xxxv, pp. 31, 591; Lippmann: *Chemie d. Zuckerarten*, Braunschweig, 1904, ii, p. 1636.

⁵ Lippman: *ibid.*, xxv, p. 257; xxxviii, p. 1232; Lippmann: *ibid.*, pp. 1636, 1637.

⁶ Creydt: *loc. cit.*

5. Experiment with Residue II.

The residue, insoluble in boiling 95 per cent alcohol, was slightly dark colored and had a slightly sweet taste. Upon qualitative tests, the residue gave almost similar reactions to those of Residue III. On similar treatment a substance resembling raffinose with a specific rotation of $+103.7^{\circ}$ was obtained. It gave 6 per cent of mucic acid on oxidation with nitric acid.

SUMMARY.

The reducing sugars of the arrowhead tubers consist of both glucose and fructose. Whether the galactose is present as such or in combination with other hexoses is not settled.

The non-reducing sugars consist of sucrose and a sugar which seems to be raffinose.

The presence of maltose, pentose and mannose, free or combined, is excluded.

THE ACTION OF RENNIN ON CASEIN.

By ALFRED W. BOSWORTH.

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The changes produced by the action of rennin in milk and solutions of casein have been the subject of many investigations. Fremy¹ was probably the first to give an explanation of this phenomenon. He believed the power to coagulate milk possessed by an extract of the mucous lining of a calf's stomach to be due to the presence of an enzyme which converted some of the lactose of the milk into lactic acid, the acid thus formed precipitating the casein.

Hammarsten² was the first to show that this coagulation of milk was due to the presence of a soluble ferment which acted directly upon the casein, producing, as he thought, two substances, the insoluble curd, *Käse*, which we call paracasein and a soluble product which he called whey-protein (*Molkeneiweiss*). He also showed that the change of casein to paracasein was independent of coagulation, the coagulation being due to the presence of soluble calcium salts.³

A great number of papers have been published upon this subject since the early work of Hammarsten.⁴ As his explanation of the action of rennin has been generally accepted as correct, most of the recent investigations have been concerned with the influence of soluble salts upon the coagulation. These investigations

¹ Fremy: *Ann. de pharm. (Liebig)*, xxxi, p. 188, 1839.

² Hammarsten: *Maly's Jahresbericht*, 1872, p. 118; 1874, p. 135; 1877, p. 158.

³ See also Arthus and Page: *Arch. de physiol.* (5th series), ii.

⁴ An excellent review of the literature with references may be found in Bulletin 56 of the Hygienic Laboratory of the Public Health and Marine Hospital Service of the United States.

have shown that the soluble salts of calcium, barium and strontium favor or hasten coagulation while salts of ammonium, sodium and potassium retard or inhibit coagulation.

Recently Van Slyke and Bosworth⁵ have shown that casein and paracasein are acids having the same percentage composition; that the molecular weight of casein is probably $8888 \pm$, while the molecular weight of paracasein is one-half that of casein; that both have a combining equivalent of 1111; that combinations of casein or paracasein with one equivalent of calcium, barium or strontium are insoluble in water while the combinations with one equivalent of ammonium, sodium or potassium are soluble and that ammonium, sodium or potassium caseinates can be changed by rennin to paracaseinates which are soluble and are precipitated by calcium chloride as calcium paracaseinates.

These facts would seem to indicate three things:

First, that rennin action consists of the hydrolytic splitting of the casein molecule into two similar molecules of paracasein; perhaps in somewhat the same manner that maltose is split into two molecules of dextrose.

Second, that, as a consequence of this cleavage it would seem to be doubtful if Hammarsten's whey-protein could be one of the products of rennin action.

Third, that rennin is not, strictly speaking, a coagulating ferment, the coagulation of paracasein being due to the fact that calcium paracaseinates are less soluble than the calcium caseinates, especially in the presence of soluble salts of calcium, barium or strontium.

This investigation was undertaken as an attempt to determine the truth of these statements. In repeating the work of Hammarsten and others a soluble substance which had not been coagulated by rennin and could not be precipitated by dilute acetic acid was always found in the filtrate. Casein solutions for such investigations have been prepared, as a general rule, by shaking pure casein with an excess of lime water or by grinding with moist calcium carbonate. The casein solutions thus obtained were made neutral to litmus and coagulated by the addition of rennin. The curds were filtered off and the filtrates examined for nitrogen.

⁵ Van Slyke and Bosworth: this *Journal*, xiv, pp. 203-236.

Soluble nitrogen was always found, but the amounts were not constant and seemed to have no relation to the amounts of casein or rennin used. In the control experiments, to which no rennin had been added, similar amounts of nitrogen which could not be precipitated by dilute acetic acid were also found.

Caseinate solutions prepared in the manner described, contain basic caseinates, either neutral or alkaline to phenolphthalein. As Robertson⁶ has shown that such caseinates in solution undergo an autohydrolysis, the following experiment was carried out in order to determine if this might account for the soluble nitrogen found.

Five grams of casein were dissolved in 250 cc. of $\frac{N}{30}$ calcium hydroxide in the presence of toluol. After complete solution of the casein portions of the solution were withdrawn at intervals and the casein precipitated by means of dilute acetic acid. The casein was filtered off and the nitrogen in the filtrates determined by the microchemical method devised by Folin.⁷ The results are as follows:

	3 hours	15 hours	24 hours
Milligrams of nitrogen in original solution.....	158	158	158
Milligrams of nitrogen not precipitated by rennin....	4.0	10.0	23.8

Results of the same nature were obtained with solutions made by grinding casein with moist calcium carbonate. The extent of this autohydrolysis, temperature being constant, depends upon time. A dry casein goes into solution very slowly and freshly precipitated casein is quite rapidly redissolved the following procedure was adopted in order to circumvent autohydrolysis.

Ten grams of pure dry casein were dissolved in 500 cc. of $\frac{N}{30}$ calcium hydroxide. The casein was then precipitated by adding about 250 cc. of $\frac{N}{25}$ acetic acid, the liquid was siphoned off, the casein washed several times with water, placed in a linen bag and squeezed as dry as possible. It was then transferred to a mortar, ground to a paste with a little water, the paste put into

⁶ Robertson: this *Journal*, ii, p. 344; see also Osborne: *Journ. of Physiol.*, xxvii, p. 398.

⁷ Folin and Farmer: this *Journal*, xi, p. 493. All nitrogen determinations made in this paper were made by this method.

a flask and 150 cc. of water, 75 cc. of lime water and some toluol were added to it. After considerable shaking the lime water became saturated with casein. By this process a solution was obtained containing a calcium caseinate neutral to litmus but acid to phenolphthalein,⁸ and containing four equivalents of base. The undissolved casein was removed by centrifuging and filtering. The amount of casein in solution was determined and the solution so diluted that each 50 cc. contained 1 gram of casein. Fifty cc. portions of this solution were withdrawn at intervals* and precipitated with acetic acid. The amounts of nitrogen found in the filtrates were as follows:

	30 min- utes	5 hours	12 hours	24 hours
Milligrams of nitrogen in original solution.....	158	158	158	158
Milligrams of nitrogen not precipitated by acetic acid.....	0.07	0.92	1.96	2.00

Casein solutions prepared in this manner gave the following reactions. *They were not coagulated by rennin.* The addition of a few drops of a 10 per cent solution of calcium chloride caused them to curdle;⁹ the addition of one drop caused no change but the subsequent addition of rennin produced coagulation. If enough hydrochloric acid was added to change the caseinate to one containing two equivalents of calcium,¹⁰ the addition of rennin caused coagulation. That this coagulation was not due to the calcium chloride formed by the acid was shown by the fact that rennin caused coagulation after all this calcium chloride had been removed by dialysis. In both instances *the coagulation removed all the nitrogen from the solution*, as is shown by the following figures:

Milligrams nitrogen in
original solution

316

316

316

Milligrams nitrogen not precipi-
tated by rennin

0.8

0.6

0.2±

⁸ Robertson: this *Journal*, ii, p. 317; Van Slyke and Bosworth: *ibid.*, xiv, p. 211-225.

⁹ Robertson: *ibid.*, ii, p. 381. Robertson believes that the addition of the common Ca ion represses the dissociation of the caseinate and thus causes precipitation.

¹⁰ Van Slyke and Bosworth: *ibid.*, xiv, pp. 211-225.

The behavior of such caseinate solutions towards rennin can be explained by the work of Van Slyke and Bosworth as follows:

A molecule of calcium caseinate containing four equivalents of base is split by rennin into two molecules of paracaseinate, each containing two equivalents of base. Such a paracaseinate is soluble in pure water but insoluble in the presence of more than a trace of a soluble calcium salt. A molecule of calcium caseinate containing two equivalents of base is split by rennin into two molecules of paracaseinate each containing one equivalent of base. Such a paracaseinate is insoluble in pure water.

The small amounts of nitrogen recovered in the filtrates in the experiments given above may be due to autohydrolysis or to proteolysis produced by the pepsin in the rennin extract used, as is indicated by the following experiment.

Into each of several flasks were placed 50 cc. of a casein solution and a little toluol. One-half of the flasks received a few drops each of rennin solution, the others being kept as controls. The contents of the flasks were examined at intervals for autohydrolysis and proteolysis. The nitrogen in the control flasks which was not precipitated by acetic acid was considered as due to autohydrolysis; while in the case of the other flasks the nitrogen not removed by filtering was considered to be due to autohydrolysis and proteolysis. By subtracting the nitrogen found in the controls from those containing rennin a fair idea as to the extent of the proteolysis might be obtained.

	30 minutes	12 hours
Milligrams of nitrogen in original solution as casein....	158	158
Milligrams of nitrogen in filtrate from rennin flasks....	3.4	18.2
Milligrams of nitrogen in filtrates from control autohydrolysis.....	0.1	2.1
Milligrams of nitrogen due to proteolysis.....	3.3	16.1

Solutions of ammonium, sodium or potassium caseinates containing two or more equivalents of base could not be coagulated by rennin, but the subsequent addition of calcium chloride caused coagulation, the curd being calcium paracaseinate. That sodium caseinate in solution was changed to sodium paracaseinate was shown by the following experiment. Rennin was added to a solu-

tion of sodium caseinate and after a short time acetic acid was added. The precipitate, after being purified and dried, was found to be paracasein.

The conclusions drawn from this investigation are as follows:

A solution of calcium caseinate neutral to litmus and free from all other salts is not curdled by rennin.

A solution of calcium caseinate acid to litmus, which contains two equivalents of base for each molecule of casein, is curdled by rennin.

Solutions of ammonium, sodium or potassium caseinates are not curdled by rennin. In such solution however the casein is changed to paracasein, the paracaseinates of these bases being soluble.

When paracasein is produced from casein by the action of rennin no other substance is formed. Two molecules of paracasein are produced from each molecule of casein as a result of this action.

Rennin is not, strictly speaking, a coagulating ferment; the coagulation being a secondary effect, the result of a change in solubilities.

Rennin action is probably a hydrolytic cleavage and may be considered the first step in the proteolysis of casein. It would follow from this that the action now attributed to rennin may be produced by any proteolytic enzyme. Work along this line is being carried out by the author.

In the light of the results reported in this paper together with those of Van Slyke and Bosworth the retarding action of soluble salts of ammonium, sodium and potassium on the coagulation of milk or casein solutions by rennin may be explained as follows. The addition of salts of these bases to milk or casein solutions results in a double decomposition whereby the calcium caseinate is changed to a caseinate of the base added. These are converted to paracaseinates by rennin, but owing to the fact that all the paracaseinates of these bases are soluble, no coagulation results.

In conclusion I wish to express my appreciation of the interest in this work shown by Dr. L. L. Van Slyke, of the Chemical Laboratory of the New York Agricultural Experiment Station, Geneva, N. Y., and Dr. Otto Folin of the Biochemical Laboratory of the Harvard Medical School, Boston, Mass.

THE FORMATION OF INDOPHENOL AT THE NUCLEAR AND PLASMA MEMBRANES OF FROGS' BLOOD CORPUSCLES AND ITS ACCELERATION BY INDUCTION SHOCKS.

PLATE I.

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In my earlier paper on the localization of the formation of colored oxidation products in the cells and tissues of the frog,¹ I described experiments in which dimethyl-*p*-diamino-benzene $C_6H_4.NH_2.N(CH_3)_2$ was used (instead of the usual unsubstituted compound $C_6H_4(NH_2)_2$) in conjunction with α -naphthol for the intracellular production of indophenol. A mixture of these two substances forms on oxidation dimethyl indophenol, a deep blue, water-insoluble compound which is deposited within the cells in the form of conspicuous granules; these show in many cases a highly definite distribution; thus in red corpuscles and leucocytes a tendency to deposition at the surface of the nuclear membrane is very constant and pronounced. This appearance is so definite that it seemed at that time to afford strong support to the view that the cell nuclei have a special relation to oxidation processes. The photographs on plate I (figs. 1 and 2) illustrate this condition in corpuscles that have lain for some minutes in a solution of α -naphthol and dimethyl-*p*-diamino-benzene in physiological salt solution. It will be seen on referring to these photographs that the granules at the boundary of nucleus and cytoplasm in the erythrocytes are larger and more densely massed than elsewhere in the cell.

There is little doubt that the regions where the indophenol is

¹ *Amer. Journ. of Physiol.*, vii, p. 412, 1901.

most densely deposited within these cells correspond to the regions of its most rapid formation, and hence to the regions of most active oxidations. Indophenol granules show an active Brownian movement when freely suspended,—as in a solution of α -naphthol and *p*-diamino-benzene undergoing spontaneous oxidation; but when the granules are deposited in the interior of the cells little or no movement is perceptible, and under these conditions they must tend to remain in the place of their formation. Direct observation shows that their aggregation at the nuclear membrane of erythrocytes is not the effect of a secondary gathering in this situation, subsequent to formation elsewhere in the cell; the first granules that make their appearance in erythrocytes after placing in the indophenol-forming solution almost always occupy this position, and it is not until later that granules appear elsewhere in the cell. The region adjoining the nuclear surface in erythrocytes appears thus to be a region of relatively rapid oxidation. Evidence that oxidative processes of this kind may show definite localization in cells acquires especial significance in view of Vernon's recent observations, according to which the rate of formation of indophenol is a fair index of the general oxidative power of the tissue;² if this is the case, the conditions of formation of this compound in cells probably correspond in the main to those of the other and physiologically important oxidations.

Further study has however convinced me that the nuclear surface is not, in general, necessarily the seat of more active oxidations than the other free surfaces or phase boundaries in cells. The oxidation process appears to be strongly influenced by the conditions at surfaces; adsorption and variations of phase-boundary potentials are probably the chief factors in this influence, as apparently in other forms of catalysis due to surface action. I find that in voluntary muscle cells the indophenol formation shows no evident relation to the scattered superficial nuclei, but appears diffused throughout the cytoplasm. Again, in frozen sections of the frog's brain and spinal cord the distribution of most active indophenol formation does not correspond to the distribution of nuclei in these tissues; the oxidation is, in fact, more active in the white than in the gray matter. These facts,

² Vernon: *Journ. of Physiol.*, xlii, p. 402, 1911.

as well as various more general considerations partly discussed below, suggest that the formation of indophenol at the nuclear surface in blood corpuscles is not to be regarded as indicating a specific relation of nuclei to oxidations; it seems more probable that it indicates the existence of a general relation of the cell-surfaces or protoplasmic phase boundaries to oxidations. There are other facts indicating this. Warburg's observations on sea-urchin eggs show that alkali may modify oxidations without penetrating the cell—apparently by altering the state of the general cell-surface or plasma membrane.³ Nernst's theory of electrical stimulation implies that the rate of oxidation in irritable tissues is increased by varying the state of electrical polarization of the semi-permeable cell surfaces; i.e., increase in oxidation is a very general consequence of electrical stimulation, whose essential condition is alteration of the electrical polarization at such surfaces.

Re-examination of the conditions of indophenol formation in frog's blood corpuscles has confirmed my former observations of a relatively rapid oxidation at the nuclear surface; the appearances indicate also that a second region of relatively rapid oxidation exists near the general outer surface of the cell, or just within the plasma membrane. It is difficult to obtain unequivocal proof of this in the case of erythrocytes, on account of the flattened shape of these cells; but when the cells are slightly crenated and are viewed in face with a high power, the granules of indophenol often show a network-like disposition which apparently corresponds to the folds of the surface; examination with the oil-immersion lens—especially when the cells are viewed edgewise—also often shows a denser distribution of granules just within the cell-surface than in the region intermediate between plasma and nuclear membranes. The region of densest deposition of granules in erythrocytes is, however, always the nuclear surface, and apparently this is the region of most active oxidation in these cells.⁴ In leucocytes a relation of oxidation to the general

³ Warburg: *Zeitschr. f. physiol. Chem.*, lxi, p. 305, 1910.

⁴ Warburg's observations on the rate of oxygen consumption by the red corpuscles of birds show an interesting parallel with those just cited (cf. Warburg: *Zeitschr. f. physiol. Chem.*, lxx, p. 413, 1911; *Munch. med. Wochenschr.*, lviii, p. 289, 1911). These cells show a much higher oxygen consumption, especially when newly formed, than the non-nucleated ery-

cell-surface is more distinct. Indophenol is formed in these cells much more rapidly than in erythrocytes, and in a mixture of α -naphthol and dimethyl-*p*-diamino-benzene solutions, of the composition given below (page 243), the surface layer of the cytoplasm of large leucocytes, as well as the region adjoining the nucleus, quickly becomes filled with large and conspicuous granules. The presence of numerous granules just within the cell-surface is highly characteristic of leucocytes that have lain for a few minutes in the indophenol-forming solution. Later the whole cytoplasm becomes densely laden and the original inequality of distribution is obscured. In some of the photographs reproduced with this article rows of granules just within the cell-surface are clearly seen (see figs. 4 and 5).

These observations show that a close relation exists between oxidations and certain intracellular surfaces or phase boundaries. It is theoretically improbable that the surfaces of semi-permeable membranes like nuclear or plasma membranes are alone concerned in these processes. The surfaces of other colloidal structures probably play a similar part in cells; thus in muscle cells it is possible that the large surface of contact between fibrils and sarcoplasm forms a region active in oxidations, although there is no direct evidence of this as yet; if so, the high oxidative activity of these cells may in part be accounted for. Increased reaction velocity in polyphasic systems with large surface extent is a frequently observed phenomenon; the catalytic action of colloidal metals has been explained by Bredig and others as an

thromocytes of mammals. Alternate freezing and thawing destroys the cytoplasm, but leaves the nuclei intact; cells thus altered show an unimpaired or even increased rate of oxidation. Evidently the general cell-surface is not essential to oxidation in these cells; if however the *nuclear* surface is the active region in birds' erythrocytes, as in those of frogs, Warburg's results may readily be accounted for. His further observation that salts like calcium, magnesium, and barium chloride influence oxidations *after*, but not *before*, destruction of the plasma membranes also receives a consistent explanation, since the plasma membranes are normally impermeable to these salts, which accordingly have free access to the nuclear membrane only after the plasma membranes are destroyed. On the other hand, phenyl, methyl, and ethyl urethanes, and hydrocyanic acid, which penetrate the plasma membranes with ease, influence oxidations equally in intact and injured erythrocytes.

instance of such surface action, and this action is very generally referred to increased concentration of the interacting substances at the phase boundaries. It is probable that the electrical condition of these surfaces also constitutes an important factor in the catalytic action.⁵

The importance of surface processes in cell activities has been widely recognized of recent years, especially since the rise of colloidal chemistry and its application to physiological problems. The character of the electrical polarization at surfaces is known to influence profoundly the adsorptions at such surfaces,⁶ and any chemical changes dependent on adsorption (as catalyses of the class referred to above) must be correspondingly influenced by the electrical condition of the surfaces. Hence chemical processes occurring in polyphasic systems must in many cases be influenced by changes in the polarization of the surfaces, to a degree which under some conditions may largely determine the general course and velocity of certain reactions; and the possibility that the chemical effects of stimulation may belong in this category thus requires consideration. According to Nernst's theory, changes in the electrical polarization of the plasma membranes form the essential condition of electrical stimulation. The same is almost certainly true of other forms of stimulation, including the normal, since all are accompanied by electrical variations whose characteristics point unmistakably to variations of phase-boundary potentials as their determining condition.⁷ The rate of oxidation in voluntary muscle cells is increased

⁵ See the communication of S. W. Young: On the Influence of Light on the Electric Potential of Bacterial and Other Suspensions, in the *Proc. of the Soc. for Exp. Biol. and Med.*, x, p. 151, 1913.

⁶ Cf. L. Michaëlis in Koranyi and Richter's *Physikalische Chemie und Medizin*, ii, 1908, pp. 347 seq.; Michaëlis and Ehrenreich; *Biochem. Zeitschr.*, x, p. 283, 1908; Höber: *Physikalische Chemie der Zelle und der Gewebe*, 1911, p. 294; Wolfgang Ostwald: Die neuere Entwicklung der Kolloidchemie in *Kolloidchemische Beihefte*, 1912, iv, p. 16. Ostwald distinguishes "electrical adsorption" from adsorption due to lowering of the surface tension at the phase boundary. In general adsorption occurs whenever the energy potential at the phase boundary is lowered by the accumulation of the dissolved substance in this region.

⁷ Cf. my paper on the relation of membrane-changes to stimulation and conduction, in *Amer. Journ. of Physiol.*, xxviii, p. 197, 1911.

many times by stimulation; and investigation of the action current of this tissue with the thread galvanometer shows that stimulation is normally associated with a rhythmical electrical variation of a definite rate; this rate varies with temperature according to the chemical temperature coefficient,⁸ i.e., shows a general parallelism with the oxidative and other chemical activity of the tissue as influenced by temperature change. An interdependence of some kind is thus indicated, and it seems likely that this interdependence is direct, and that the polarization changes form the immediate condition of the oxidations. The mechanism of intracellular oxidations is still largely obscure, and it is admitted by most biological chemists that the known properties of oxidases cannot account for the character and high velocity of the intracellular oxidations. No enzymes or combinations of enzymes and co-enzymes can accelerate the oxidation of sugar or lactic acid to anything like the degree required. Such facts suggest that the oxidases are not the main factors in intracellular oxidations, but play a merely accessory part; their presence may be favorable to rapid oxidation (by diminishing resistance), but the essential determining conditions appear to be of a quite different kind. Some feature or features of the structural organization of the cell must be fundamentally concerned, since destroying cell structure always greatly diminishes the oxidative activity of the tissue.⁹ Now the semi-permeable membranes of irritable tissues constitute the structural elements which are primarily essential to electrical stimulation, and hence to the increase of oxidation which is associated with stimulation. According to Nernst's theory, stimulation involves changes in the electrical polarization of these membranes. Such changes of surface polarization must involve corresponding changes in the polarization of the other polarized surfaces within the cell; i.e., the conditions of equilibrium of the double layers at these intracellular surfaces will be altered by altering the polarization of the general cell-surface, and their state of polarization will undergo corresponding change; hence the normal variations of polarization at the cell-surface accompanying stimulation must involve similar changes at the active surfaces throughout the cell, and it is these polarization changes

⁸ Cf. Piper: *Arch. f. (Anat. u.) Physiol.*, 1910, p. 207.

⁹ Cf. Fletcher and Hopkins: *Journ. of Physiol.*, xxxv, p. 287, 1907.

—according to the hypothesis which I am presenting—that mainly determine the increase in the rate of oxidation. Similar or related views have been tentatively put forward by a number of investigators,¹⁰ but hitherto little or no experimental evidence in their support has been adduced. The experiments about to be described show that the formation of indophenol in leucocytes—a process largely dependent on surface action as already described—can in fact be markedly accelerated by the passage of induction shocks through a suspension of corpuscles on a slide.

EXPERIMENTAL.

In the following experiments the blood corpuscles of the frog have been used. Immediately after shedding, the blood is mixed with a solution consisting of nine volumes $\frac{N}{3}$ NaCl plus one volume $\frac{N}{3}$ potassium oxalate; this solution prevents clotting and breakdown of leucocytes. The suspension of corpuscles is then mixed with the solution of the indophenol-forming reagents in slightly alkaline physiological salt solution. The solution which I have chiefly used is made (shortly before using) by mixing a saturated solution of α -naphthol in alkaline isotonic NaCl solution ($\frac{N}{3}$ NaCl containing $\frac{N}{100}$ Na_2CO_3) with a 0.5 per cent to 1 per cent solution of Merck's dimethyl-*p*-diamino-benzene in $\frac{N}{3}$ NaCl. These concentrations are favorable to the rapid formation of indophenol within the cell. With more dilute solutions the formation of indophenol is slower and its acceleration by induction shocks is less evident. Usually equal volumes of these solutions (1 cc. of each) are mixed in a test tube, and the resulting solution is then mixed intimately with a few drops of the suspension of corpuscles either in a watch glass or on a slide. This preparation may be mounted and examined at once or after the desired interval of time has elapsed. Leucocytes left in this solution (with sufficient access of oxygen) become deeply laden with indophenol granules in a few minutes. In erythrocytes the deposition of granules is more gradual; in these cells the first formed granules typically appear at the nuclear surface, as already described. Figures 1 and 2 are photographs of corpuscles taken after remaining for twenty-five and fifty-two minutes respectively

¹⁰ Cf. Warburg, 1910, *loc. cit.*

in a solution of the above composition. By this time the leucocytes are so densely laden with indophenol as to prevent the appearance of mere clumps of granules. The erythrocytes are much less deeply impregnated and the contours of the nuclei are plainly marked by the deposition of granules.

The accelerating influence of induction shocks on this oxidation may be demonstrated as follows. Immediately after mixing with the reagent, as above described, a few drops of the suspension of blood corpuscles are mounted on a specially prepared slide crossed by two fine parallel platinum wires about 2 cm. apart. The slide is already in position on the microscope stage; the wires are connected to the secondary coil of an inductarium (Porter's model) arranged for "tetanizing" currents, and a succession of shocks is passed through the preparation. If a suspension of corpuscles so treated is compared with a control consisting of a portion of the same suspension similarly mounted on a precisely similar slide (so as to have conditions of oxygen supply, etc., as nearly alike as possible), but not subjected to induction shocks, a marked difference soon becomes evident. Within a brief period, varying according to the strength of the shocks and concentration of the solution, the leucocytes in the "stimulated" preparation are seen to be plainly more deeply impregnated with indophenol than those of the control. The contrast is very striking if the conditions of concentration, number of corpuscles,¹¹ and strength of stimulation are favorable (see plate I, figs. 3-5). The difference is not due to the oxygen liberated at the wires serving as electrodes, for the acceleration of indophenol formation is seen throughout the entire region between the electrodes.

The following protocols of experiments will illustrate the character of these observations.

November 4, 1911. The blood of a large frog was mixed with an approximately equal volume of oxalate-containing sodium chloride solution. A drop of this suspension of corpuscles was placed on a slide provided with platinum wires; a drop of the indophenol-forming mixture was placed in

¹¹ Too dense a suspension of corpuscles is unfavorable to this demonstration, probably because the oxygen is too rapidly abstracted by the erythrocytes, leaving less available for oxidation in the leucocytes.

contact with the drop of suspension and a cover glass was put in place. Induction shocks were then passed.

In six successive experiments of this kind the leucocytes were found to form indophenol granules more rapidly under this treatment than in the untreated control. In some experiments the contrast at the end of two or three minutes was very striking; the leucocytes near the edge of the cover glass, where most oxygen was available, being then so deeply laden as to appear like masses of granules, while in the control only a moderate deposition of granules had taken place.

Numerous other experiments showed similar results. Usually the suspension of corpuscles and the indophenol-forming mixture were mixed before mounting. The following typical experiments are cited in further illustration.

November 15, 1911. The suspension of corpuscles was mixed with the freshly prepared indophenol-forming solution in a watch-glass and then mounted on the slides with platinum wires. Induction shocks from a Porter's inductorium with three Edison primary cells and coils 3 cm. apart were passed through the preparation. Nine experiments were performed under these conditions. The following results are typical.

EXPERIMENT 3. At 12.11 the current is started through the preparation. The granules appear rapidly in the leucocytes; at 12.16 the field of the stimulated preparation is full of intensely colored leucocytes; in the control, although many leucocytes are well laden with indophenol, the number of deeply impregnated cells and the degree of loading are much less. By 12.20 the erythrocytes of the stimulated preparation also show a distinctly deeper tint than those of the control.

EXPERIMENT 4. Current started at 12.37. The result is similar to the above; in three minutes there is a decided contrast between experiment and control. At 12.43 the erythrocytes also show considerable indophenol; the nuclear membranes appear to be impregnated with the dye, and stand out sharply. Some leucocytes show granules at the nuclear surface, and others show rows of granules just within the cell-surface.

EXPERIMENT 8. Conditions and results the same. The contrast between the microscopic fields of experiment and control under a low power is striking; eight minutes after starting the current the field of the stimulated preparation appears dotted at intervals with intensely blue-black dots corresponding to the leucocytes; in the control the leucocytes stand out much less conspicuously.

The above records are sufficient to indicate the general character of these observations. It will be noted that the induction shocks used in the above experiments are of considerable strength. Weaker shocks also accelerate the rate of oxidation in leucocytes but less obviously. It is also noteworthy that the effect on the

erythrocytes is comparatively slight; the oxidative activity of these cells, besides being much less energetic than that of the leucocytes, is less easily influenced by electrical stimulation, although after passing strong shocks for some minutes there is an appreciable increase in the formation of indophenol. The much higher oxidation rate and greater responsiveness of the leucocytes correspond to the more active part which these cells play in the organism; thus it is not surprising to find that their oxidation rate—and hence their general rate of metabolism and output of energy—is more easily altered by changes of condition. Leucocytes, although relatively inert as compared with (e.g.) muscle cells, have in fact many of the characteristics of irritable cells. Hamburger has shown that their phagocytic activity may be increased artificially by a variety of substances, including calcium salts and lipid solvents in low concentration.¹² The conditions under which oxidations are accelerated in these cells are thus probably of the same essential kind as those prevailing in irritable cells in general.

The possibility naturally suggests itself that various chemical processes other than oxidations may be similarly influenced by changes of polarization at the intracellular surfaces. The formation of indophenol is in fact a synthesis involving dehydration, as well as an oxidation, but probably no especial significance attaches to this fact. What seems to be significant is that the chemical process occurs in close relation to the intracellular surfaces, and is influenced by changes in the electrical polarization of these surfaces.¹³ It seems likely that conditions of this kind play an important general rôle in cell metabolism. The precise conditions of the above electrochemical effect are largely obscure to me at present. The analogy with photochemical action seems

¹² Hamburger and Hekma: *Biochem. Zeitschr.*, ix, p. 275, 1908; Hamburger, de Haan and Bubanovic: *Archives Néerlandaises des Sciences Exactes et Naturelles*, Ser. III, B., i, p. 1, 1911. Hamburger's recent book: *Physikalisch-chemische Untersuchungen über Phagozyten*, Wiesbaden, J. F. Bergmann, 1912, contains a full account of these and related observations.

¹³ The formation of peroxides may possibly be favored by this process, just as the formation of ozone and hydrogen peroxide is favored by the passage of electric discharges through moist air. There is good evidence that peroxides play an important—though it may be a supplementary—rôle in protoplasmic oxidations.

perhaps the most illuminating. In both cases increased displacement, or increased range of movement, of electrons—due to the electro-magnetic effect of the ether waves, or of the variation of surface polarization—and consequent facilitation of the electron transfers that condition the chemical change, are probably the fundamental factors of the action. Further discussion of this matter would, however, be inappropriate in this place.

SUMMARY.

1. In frogs' blood corpuscles the formation of indophenol by the intracellular oxidation of a mixture of α -naphthol and dimethyl-para-diamino-benzene takes place most rapidly in the immediate neighborhood of the nuclear and plasma membranes. The conditions at the surfaces of these structures are thus particularly favorable to rapid oxidations.

2. The passage of induction shocks through a suspension of corpuscles in the indophenol-forming solution accelerates this oxidation; this effect is slight in erythrocytes and well marked in leucocytes.

3. These observations indicate, in general, (a) that the intracellular surfaces or phase boundaries play an active rôle in oxidations in living cells, and (b) that variations in the electrical polarization of these surfaces form an important factor in these oxidations, and especially in the increase of oxidations following stimulation.

EXPLANATION OF PLATE.

The photographs were made by Dr. H. G. Kribs, of the Zoölogical Department, University of Pennsylvania, to whom I take pleasure in expressing my best thanks. The magnification is about 500 diameters. The exposures were brief (5 to 10 seconds) and were made within one to two minutes after the stimulating current had ceased. The corpuscles shown in these photographs are entirely typical of the conditions throughout the preparation.

Fig. 1. Showing spontaneous formation of indophenol in corpuscles. The cells were photographed after lying undisturbed for twenty-five minutes in the indophenol-forming solution. The densely laden central corpuscle is a leucocyte. The round dots scattered over the field are indophenol granules; the halo about each is due to Brownian movement.

Fig. 2. Corpuscles from a similar "unstimulated" preparation after fifty-two minutes in the solution. The round black mass just above the middle of the figure is a leucocyte. The rows of granules at the nuclear surface in the erythrocytes are typical.

Fig. 3. Stimulated preparation. The corpuscles were placed in the freshly prepared indophenol-forming solution and one-half minute later induction shocks were passed for three minutes through the preparation (mounted on a slide as described in the text. Two Edison primary batteries; Porter's inductorium with coils 2 cm. apart). Exposure was made one minute after current ceased. Four leucocytes are shown in the field; the one at the left is less densely laden than the other three.

Fig. 3a. Control of figure 3. The corpuscles were mounted as in the experiment of figure 3 but not subjected to induction shocks. Exposure was made after remaining for the same length of time (five and one-half minutes) in the solution. Six leucocytes in the field, all showing considerable formation of indophenol, but much less than in the stimulated corpuscles.

Fig. 4. Conditions the same as in the experiment of figure 3. Three deeply laden leucocytes in the field.

Fig. 4a. Control of figure 4 (in the solution for the same length of time, but unstimulated). Two leucocytes seen, showing much less indophenol formation than those of figure 4.

Fig. 5. Conditions like those of the preceding experiments, but shocks passed for only two minutes. Exposure four and one-half minutes after placing in the indophenol-forming solution. Two leucocytes are seen, showing typical conditions.

Fig. 5a. Control of figure 5. In solution for the same length of time before exposure. Two typical leucocytes shown.



FIG. 5



FIG. 5a

THE DOMINANCE OF ROQUEFORT MOLD IN CHEESE.¹

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(From the Storrs Agricultural Experiment Station.)

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Roquefort and certain related types of cheese contain practically pure cultures of Roquefort mold (*Penicillium roqueforti* Thom). Roquefort in its manufacture is inoculated with this mold prepared in pure culture. Gorgonzola is not. Stilton is not. Yet these latter cheeses contain this mold in fairly pure condition. What factors lead to the dominance of this mold in such cheeses, whether it is put in during the making period or not? Study of large numbers of milk cultures shows clearly that nearly all milk contains spores enough of various species of *Penicillium* as well as other molds to admit of a wide variety of mold colonies in any cheese provided conditions are favorable for such molds to grow. Clearly then there are factors present which favor this species in competition with the many other forms which are initially present in milk and which have been shown to grow fully as readily upon milk and milk products as does Roquefort mold. Preliminary cultural experiments by one of us, together with the work of Clark upon Emmental cheese directed our attention to the gases of the cheese as a probable factor in this dominance.

Clark² studied the gases of Emmental cheese as an index to the biological factors concerned in its ripening. This variety of cheese has attracted special attention because of the scientific interest and the economic importance attached to "eye" formation. A cheese having the texture of Swiss is quite impermeable to gases and the gases produced are retained in the cheese near

¹ Published by permission of the Secretary of Agriculture and the Director of the Storrs Agricultural Experiment Station under whose coöperative arrangement the work was done.

² Clark: U. S. Dept. of Agric., Bureau of Animal Industry, Bulletin 151, 1912.

their place of origin. The origin of the gases is therefore the problem of chief interest. A cheese having the texture of Roquefort presents an entirely different problem. *Penicillium roqueforti*, which is probably the chief factor in the ripening of this variety of cheese, is not an anaerobic organism and in order that enough oxygen may diffuse through the cheese mass to sustain the growth of this mold, it is made with as open texture as possible and about forty holes the size of a knitting needle are punched through it by means of the "prickle machine." This investigation was undertaken with the hope of getting some definite information concerning the gases within the cheese and their relation to the growth of *P. roqueforti*.

Method of collecting the gas.

The apparatus used in collecting the gas is shown in fig. 1. The puncturing part of the apparatus consisted of a glass rod *A* which fits snugly into the glass tube *B*. The tube *B* was cut in two about one inch from the lower end. The lower section was sealed fast to *A* and the upper section could be slid up or down. With the two sections of *B* together, the puncturing part of the apparatus was pushed nearly through the cheese. The upper section of *B* was then pulled up into the position shown in the figure, the adjoining section of capillary tubing connected and cock *C* closed. The entire cheese was then coated with paraffine by repeated dipping. The dipping was conducted slowly to prevent heating the interior of the cheese. When the paraffine coat had attained a thickness of about one inch, the entire cheese was imbedded in paraffine nearly at the solidifying temperature. When the cheese was thoroughly cooled the apparatus was connected as shown in the diagram. A calcium chloride tube *D* was placed between the cheese and the gas-collecting tube *G* to intercept any water that might be drawn up with the gas. The collecting tube *G* had a capacity of 230 cc. *E* led to a manometer and *K* to a water suction pump. With the cock *C* closed, the apparatus was exhausted to the limit of the pump which was about 25 mm. Cock *H* was then closed, and when assured that the apparatus was perfectly tight cock *C* was opened and the gas within the cheese allowed to expand into the collecting tube. When the pressure almost or entirely ceased to rise, cock *F* was closed and the collecting tube disconnected. The amount of oxygen and nitrogen from the air remaining in the collecting tube, was determined by exhausting to exactly the same pressure as that at which the gas was collected, and measuring the air by displacement with mercury.

This method of collecting the gas, although simple, appears to give accurate results. The amount of oxygen is no greater than is really to be expected in a cheese in which gaseous diffusion is as free as in Roquefort. The most probable error is due to the

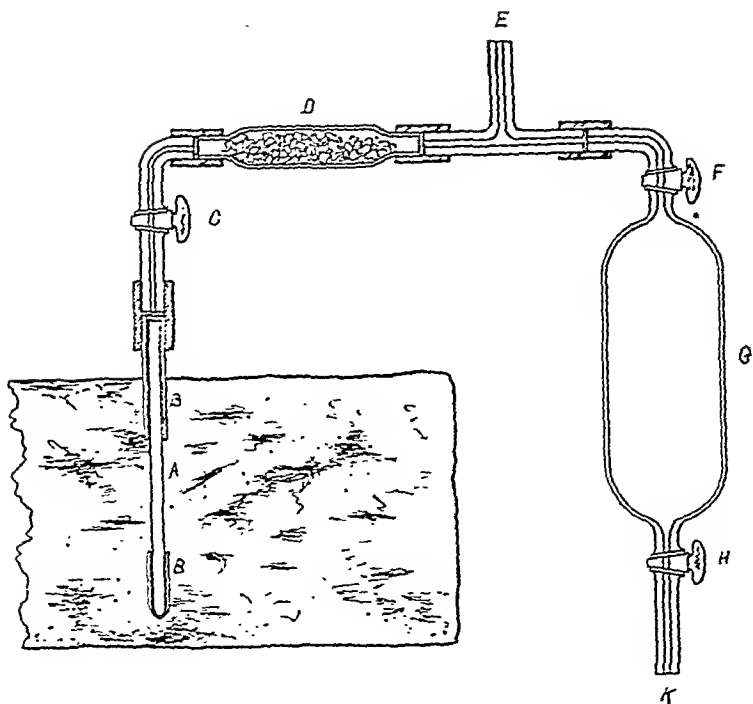


FIG. 1. APPARATUS FOR COLLECTING GAS.

film of air between the cheese and the first layer of paraffine. At least twenty-four hours elapsed between the time the first coat of paraffine was applied and the time the gas was collected. The respiration of the microorganisms on the surface of the cheese and the diffusion of the gases throughout the cheese would greatly reduce this error. The ingenious apparatus devised by Clark for the study of Swiss cheese is not adapted to a cheese having the texture of Roquefort.

The gas was displaced from the collecting tube with mercury, the carbon dioxide determined by absorption over potassium hydroxide and the oxygen by absorption over phosphorus. The residual gases of cheeses 2 and 5 were burned with air in a mercury pipette by means of an electrically heated, platinum spiral. Neither sample showed the presence of hydrocarbons. The gas from cheese 5 gave a contraction which would have corresponded to 1.0 per cent of hydrogen. The residual gas of cheese 3 was

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tested for hydrogen by repeated passing over palladium at 90°C. The gas showed no reduction in volume. The conclusions were therefore drawn that the residual gas consisted chiefly of nitrogen, that it was free from hydrocarbons but may have contained small amounts of hydrogen. It is reported as nitrogen in the following table.

TABLE I.
Analyses of the gases of Roquefort cheese.

BRAND	CC. OF GAS AT 0°C. 760 MM.					PER CENT			REMARKS
	Gas collected	Gas analyzed	CO ₂	O ₂	N ₂	CO ₂	O ₂	N ₂	
1. Louis Rigal	18.21	18.21	5.07	0.44	12.21	27.84	2.42	69.74	Slight growth of mold.
2. Société.....	123.60	44.31	11.19	1.48	31.64	25.25	3.34	71.41	
3. Veritable Roquefort	81.73	81.73	18.07	5.72	57.94	22.11	7.00	70.89	Very ripe and moldy.
4. Experimental cheese	88.25	80.29	32.88	3.64	43.77	40.95	4.53	54.52	Six days old.
5. Experimental cheese	59.80	59.80	12.64	3.24	43.92	21.14	5.42	73.44	Seven weeks old.

From a study of these results it appears that ripening is accompanied by a process of respiration which results in the disappearance of oxygen and the production of an equivalent amount of carbon dioxide. In the early period of ripening the carbon dioxide from this source is augmented by the carbon dioxide produced by bacteria which decompose milk sugar. During this period the carbon dioxide is much higher and the nitrogen lower than would result from a simple process of respiration. The diffusion of gases tends to reduce this excess of carbon dioxide after the disappearance of milk sugar and the mixture of gases approaches the composition which would result from a process removing the oxygen from the air and producing an equivalent amount of carbon dioxide. The percentage of oxygen is always low and any aerobic organism which thrives within the cheese must be capable of obtaining its oxygen from a very dilute atmosphere of oxygen.

Various investigations have demonstrated that molds will not

grow in an atmosphere of carbon dioxide or of hydrogen. Similar results have been reported already in this investigation.³ By these cultures it was further shown by the removal of the inhibiting gas that the spores present were not injured by either gas since all species developed quickly after the gases were removed. It is physically impossible for molds such as *Penicillium* to develop normally within a dense mass of substratum. Colonies develop only near and upon the surface of the culture medium although the vegetative hyphae of some species may extend into the mass a distance of 1 cm. or even farther. In cheese, however, hyphae can rarely be demonstrated 3 mm. below the surface. The presence of hyphae in deeper areas not connected with a surface colony raises a presumption of the presence of air spaces. Bitting has shown that *Penicillium expansum* will not grow in a vacuum, thus showing conclusively its dependence upon free oxygen. In seeking the explanation of the dominance of *Penicillium roqueforti* under the conditions shown to exist in Roquefort cheese it became desirable to study the effect of varying percentages of carbon dioxide upon a series of species. In the light of previous work, the carbon dioxide is regarded by the authors as an inert gas serving to dilute the atmospheric air. Increased percentages of this gas, therefore, would reduce the amount of available oxygen proportionally. A critical percentage is not to be expected but rather a gradual reduction of activity as the amount of oxygen present becomes reduced so that growth will be gradually decreased and ultimately stopped. For this purpose cultures of twenty-two species of *Penicillium* and five species of *Aspergillus* were made in Czapek's solution⁴ agar slanted in test tubes in the ordinary way. During several years of work this medium has been found to give good average colonies of all the species used. A control set was carried under ordinary atmospheric conditions as a basis for comparison of results.

³ Thom: U. S. Dept. of Agric., Bureau of Animal Industry, Bulletin 118, p. 90.

⁴ Czapek's solution as given by Dox, A. W., U. S. Dept. of Agric., Bureau of Animal Industry, Bulletin 120: 1000 cc. of water, 0.5 gram magnesium sulphate, 1 gram dipotassium phosphate, 0.5 gram potassium chloride, 2 grams sodium nitrate, 0.01 gram ferrous sulphate, 30 grams cane sugar and 15 grams agar. The author has substituted dipotassium phosphate for monopotassium phosphate as used by Dox because he prefers a neutral solution.

First experiment. Three sets of cultures were prepared. One was kept in a crate upon the table as a control. The other sets were put into two Novy culture jars and sealed up for nineteen days. All three groups were kept close together upon the laboratory table in diffused light, and at ordinary room temperatures. When examined the control series showed good normal colonies of the type already familiar from repeated study. Samples of the air in the Novy jars were removed and analyzed for carbon dioxide. In jar No. 1, this analysis showed 24.8 per cent, and in No. 2, 24.9 per cent of carbon dioxide. The cultures were then removed and compared with the control cultures. Following a practice previously used by one of us⁵ a fully normal colony is designated in the tabulation of results as 1.0, and the observer's judgment as to amounts less than normal is expressed in tenths. To one who has cultivated these species many times the figures given in the table suggest the following interpretation. Species capable of developing normally within a few days show fully developed colonies, with perhaps one exception. *P. divaricatum* seems to be affected very quickly by the carbon dioxide since it shows about the amount of growth ordinarily produced in four days. Species requiring a week or more for normal growth ordinarily are retarded and in some cases prevented from reaching fully normal appearance. The species of *Aspergillus* except *A. fumigatus* were strongly reduced. In general the cultures had a retarded appearance resembling the appearance of colonies grown under cold conditions. Colonies nineteen days old had the appearance common to colonies seven to eight days old. The activities of the organisms seemed to be arrested as shown by the fresh bright colors and the lack of the shades of color both in spores and in mycelium usually seen in old cultures.

Second experiment. Since the jars used in the first experiment showed approximately 25 per cent of carbon dioxide at the close of the experiment, that figure was taken as a starting point for the second set of cultures. Two jars were prepared as before to contain the same set of species and were permitted to grow for eight days. The gases present were then analyzed.

	CO ₂ in beginning	CO ₂ at end
Jar 1.....	24.8	37.7
Jar 2.....	23.6	37.8

⁵ Thom: *loc. cit.*

Examination of the cultures showed a reduction of vigor for most species. However, a number of forms as reported in the table show nearly normal colonies at the end of the experiment. Certain species are greatly reduced, but the number of strong colonies includes a considerable variety of species.

Third experiment. The Novy jars were prepared again with approximately 50 per cent of carbon dioxide. The cultures stood seven days and samples of the air were then analyzed.

	CO ₂ at beginning	CO ₂ at end
Jar 1.....	49.0	52.0
Jar 2.....	48.0	46.0

Although there must have been a considerable production of carbon dioxide the percentage of this gas at the end of the experiment was very nearly the same as at the beginning. This is due to the absorption of carbon dioxide by the culture media.

The difference in the figures for carbon dioxide had no appreciable effect upon the cultures as recorded separately. The figures given in the table are the average. In this list, *P. roqueforti* and *P. expansum* give the best growth, but four colonies (0.5 or over) of *P. duclauxi*, *P. camemberti*, *P. biforme*, and *P. rugulosum* were produced. Even with *P. roqueforti* the colony failed to show the usual abundance of green conidia. It is evident that 50 per cent carbon dioxide is sufficient to eliminate some species and reduce the others but the ubiquitous and omnivorous *P. expansum* grew nearly as well as *P. roqueforti* under these conditions.

Fourth experiment. The series was prepared a fourth time with carbon dioxide as 75 per cent of the mixture of gases used. The jars stood eight days. When examined carbon dioxide was 69.2 per cent of the mixture in the one jar successfully sampled. Roquefort mold alone of the species showed fair colonies (0.5 or over). *P. duclauxi* showed some colored fruit and the others germination with more or less submerged mycelium only.

The detailed results of all four experiments are given in table II.

Comparison of these cultural results with the data from gas analysis indicates that *P. roqueforti* alone is capable of growing to any marked extent under the conditions regularly found in Roquefort cheese. The activity of this species even is greatly reduced by the scarcity of oxygen. Inspection of the gas analysis table shows that the cheese containing 7 per cent of free oxygen had produced far more mold than the others. Certain analyses

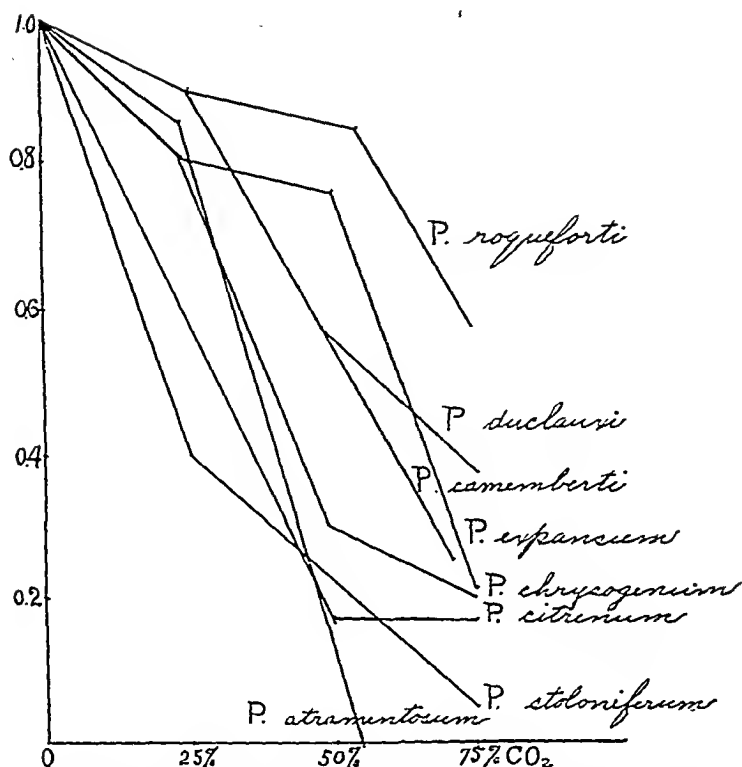
TABLE II.

Cultures of Penicillium and Aspergillus grown in Novy jar with mixture of air and carbon dioxide.

(Normal colonies designated 1.0, successive reductions in growth represented in tenths except that germination of spores alone is designated 0.1 and any further growth by increased figures.)

	CONTROL	CO ₂ 0 AT START 25 PER CENT AT END	CO ₂ 24 8 PER CENT AT START 31.7 PER CENT AT END	CO ₂ 49 PER CENT AT START 52 PER CENT AT END	CO ₂ 75 PER CENT AT START 60.2 PER CENT AT END
<i>P. camemberti</i>	1.0	1.0	0.9	0.5-6	0.2
<i>P. claviforme</i>	1.0	0.8	0.5	0.2	0.1
<i>P. granulatum</i>	1.0		0.85	0.35	0.2
<i>P. italicum</i>	1.0	0.8	0.9	0.45	0.2
<i>P. expansum</i>	1.0	0.9	0.8	0.75	0.2
<i>P. citrinum</i>	1.0	0.8	0.6	0.15	0.15
<i>P. purpurogenum</i>	1.0		0.4	0.2	0.2
<i>P. roqueforti</i>	1.0	1.0	0.9	0.85	0.55
<i>P. duclauxi</i>	1.0		0.9	0.55	0.35
<i>P. 24</i>	1.0	1.0	0.8	0.35	0.15
<i>P. chrysogenum</i>	1.0	1.0	0.9	0.3	0.2
<i>P. stoloniferum</i>	1.0	1.0	0.4	0.2	0.1
<i>P. divaricatum</i>	1.0	0.6	0.4	0.35	0.25
<i>P. atramentosum</i>	1.0	1.0	0.85	0.1	0.0
<i>P. biforme</i>	1.0		0.8	0.55	0.35
<i>P. 43</i>	1.0	0.8	0.3	0.15	0.1
<i>P. spinulosum</i>	1.0	1.0	0.6	0.35	0.15
<i>P. rugulosum</i>	1.0	1.0	0.7	0.6	0.1
<i>P. glaber</i> (Cit.).....	1.0	1.0	0.7	0.25	0.1
<i>P. 2479</i>	1.0	1.0	0.6	0.45	0.2
<i>P. 2481</i>	1.0	0.3	0.4	0.15	0?
<i>A. flavus</i>	1.0	0.5	0.7	0.35	0.2
<i>A. niger</i>	1.0	0.6	0.6	0.15	?
<i>A. ochraceus</i>	1.0	0.3	0.3	0.15	0.15
<i>A. fumigatus</i>	1.0	1.0	0.8	0.25	0.15
<i>A. flavus</i> . var....	1.0	0.6	0.7	0.4	0.2

not included suggest that the percentage of oxygen frequently falls even lower, especially in freshly made cheeses. If such cheeses are paraffined or closely covered with tin-foil no increase in oxygen is probable. In experimental work many cheeses have been produced which were open enough within to favor mold growth but



Curves showing relative activity of species beginning at 1.0 for normal and control culture and showing the relative reduction of activity with mixtures containing 25, 50 and 75 per cent carbon dioxide.

in which no growth developed in many months. Abundant mold spores were present. These experiments indicate that the failure to ripen was due to the scarcity of oxygen within these cheeses. Success in ripening cheeses of the Roquefort group will depend upon controlling the gas content of the cheeses within the range of dominance of the typical Roquefort species of *Penicillium*. Under these conditions the growth of *P. roqueforti* while slow greatly exceeds that of any other species studied. In actual practice, cultures made from many specimens of Roquefort of different brands, also from Gorgonzola and Stilton, have shown frequently pure cultures of this species while the few species found as contaminations have formed a very small part of the flora.

SUMMARY.

Analysis of the gas collected from the air spaces in Roquefort cheese (both imported and made experimentally) shows that the percentage of free oxygen is low, in no case among those tabulated rising above 7 per cent.

Carbon dioxide appears in proportions ranging from 21 to 40 per cent. The percentage of carbon dioxide is highest in comparatively fresh-made cheese where it arises both from respiration of the microorganisms present and as a by-product of the decomposition of lactose by bacteria. From the maximum figure the percentage falls slowly by diffusion.

No hydrocarbons were found. Hydrogen if present was in very small amount.

Cultures of twenty-two species of *Penicillium* and five species of *Aspergillus* grown in a Novy culture jar for nineteen days produced an atmosphere containing approximately 25 per cent of carbon dioxide. Cultures of the same species in the same jar with an initial mixture of air and 25 per cent of carbon dioxide showed 37 per cent of carbon dioxide at the end of eight days with marked reduction in the activity of certain species.

Cultures of the same series of fungi in jars with approximately 50 per cent of carbon dioxide in the initial mixture left the mixture approximately unchanged at the end of seven days. Fungous growth was stopped for certain species, greatly reduced for all, but six of the species tested produced very considerable growth.

Cultures of this series of species grown for eight days in jars with 75 per cent of carbon dioxide in the initial mixture showed that *Penicillium roqueforti* alone was able to produce fairly strong colonies in this mixture.

A mixture of 75 per cent of carbon dioxide with air gives approximately 5 per cent of free oxygen. The close correspondence between the results of gas analysis and comparative culture, indicates that the low percentage of oxygen in the open spaces within the cheese accounts for the dominant activity of *Penicillium roqueforti* in Roquefort and related types of cheese.

NOTE ON THE VOLATILITY OF SULPHURIC ACID WHEN USED IN VACUUM DRYING.

By H. C. GORE.

(From the Bureau of Chemistry of the U. S. Department of Agriculture,
Washington, D. C.)

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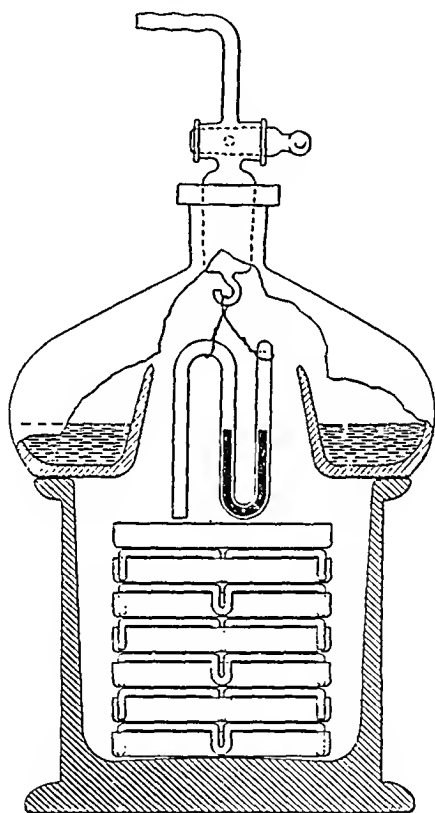
Highly perfected methods for drying perishable tissues and foods have been devised by Benedict and his coworkers,¹ and by Trowbridge,² and it is anticipated that these methods will soon come into general use, as pumps of high capacity giving high vacua are now generally available. In all of these methods sulphuric acid is used as the desiccant. Though possessing a marked advantage by virtue of its property of absorbing vapors of alcohol, ether and other substances as well as water vapor, it possesses the disadvantage of becoming coated over with a surface layer of dilute acid so that it is necessary to agitate the container gently at intervals to promote rapid drying. In addition, its corrosive nature marks it as a reagent the use of which is to be avoided wherever possible. In the course of experiments in drying in high vacuum over sulphuric acid the writer obtained indications that sulphuric acid is sensibly volatile under these conditions; for example, it was observed that flour so dried darkened perceptibly. No idea was had however of the extent to which the acid is volatile. Accordingly the experiment described below was made. The data obtained give an idea of the rate at which sulphuric acid will distil in vacuum of the order of 1 mm.

The experiment consisted in exposing powdered potassium hydroxide in a Hempel desiccator containing sulphuric acid for a long time at room temperatures at a vacuum of somewhat less than 1 mm. The details are as follows:

¹ Benedict and Manning: *Amer. Chem. Journ.*, xxvii, p. 340, 1902; *Amer. Journ. of Physiol.*, xiii, p. 309, 1905.

² U. S. Department of Agriculture, Bureau of Chemistry, Bulletin 122, p. 219.

A Hempel desiccator well cleaned and dried was charged with 200 cc. of 95 per cent C. P. sulphuric acid taken from a freshly opened bottle. A mercury manometer was suspended from the hook attached to the stop-cock in the cover by means of a platinum wire. In the lower part of the desiccator was placed a series of Petri dishes resting one upon the other, separated from each



ARRANGEMENT OF PETRI DISHES, MANOMETER, AND SULPHURIC ACID IN HEMPEL DESICCATOR.

other by glass triangles. The corners of the triangles were bent down to prevent the dishes from touching the sides of the desiccator. The arrangement is shown in the accompanying figure. Just before being placed in position each dish was charged with 5 to 10 grams of potassium hydroxide spread out in a thin layer. The ground edge of the desiccator and the stop-cock were lubricated with a mixture of vaseline and gum chicle. With the excep-

tion of a small portion of this lubricant which collected about the inner edge of the ground surface, no organic matter was present in the desiccator. The powdered potassium hydroxide used was so free from sulphates that on acidifying a test portion, adding solution of barium chloride and digesting on the steam bath, a faint, almost invisible precipitate collected in the center of the beaker on stirring.

A May-Nelson pump was used in exhausting. The vacuum obtained was slightly less than 1 mm. and remained unchanged from May 21, 1912, to January 13, 1913, 237 days. The desiccator was then opened and sulphate determined in the potassium hydroxide in each dish.

The results are given in the following table.

NUMBER OF DISH*	1	2	3	4	5	6	7	TOTAL
Sulphuric acid distilled, gm.....	0.2133	0.0440	0.0169	0.0061	0.0072	0.0026	0.0029	0.2930

* Numbered from top to bottom.

The amount of barium sulphate obtained in a blank on the reagents was not weighable. In 237 days therefore 0.2930 gram of sulphuric acid distilled from the acid in the cover of the desiccator and collected in the potassium hydroxide. The area of sulphuric acid exposed was about 188.5 sq. cm. The total loss was therefore 1.236 mgm. per day or 0.00656 mgm. per sq. cm. per day.

Experiments at the Bureau of Chemistry in drying samples of fruits in high vacuum show that lime may be successfully substituted for sulphuric acid.

THE RACEMIZATION OF PROTEINS AND THEIR DERIVATIVES RESULTING FROM TAUTO- MERIC CHANGE. PART II.

THE RACEMIZATION OF CASEIN.

BY H. D. DAKIN AND H. W. DUDLEY.

(*From the Herter Laboratory, New York.*)

(Received for publication, June 10, 1913.)

In a recent communication¹ one of us has reported experiments on the racemization of gelatin by dilute alkali at low temperatures and shown that the process is well explained by assuming a keto-enol tautomerism of the $-\text{CH}-\text{CO}-$ groups in the protein complex, and further that the optical activity or inactivity of the amino-acids obtained by hydrolysis of this racemized protein may be indicative of the positions of the respective amino-acids in the molecule.

In the hope of gaining further insight into the structure of proteins this work is being extended to other proteins and peptides and the object of this paper is to describe experiments made on the action of dilute alkali on casein.

A 10 per cent solution of casein in $\frac{3}{4}$ sodium hydroxide was digested at 37°C. until the rotation fell to a constant value. On acidifying this solution "racemized casein" was precipitated and finally obtained as a powder closely resembling ordinary casein. On analysis it was found to have C = 53.5, N = 12.5, H = 7.03 per cent, while a typical analysis of ordinary casein is C = 52.9, N = 15.6, H = 7.05 per cent. Qualitative tests of the racemized product revealed a very slight phosphorus content but a fair amount of sulphur.

Simultaneously with racemization of the protein, hydrolysis also took place, so that on saturating the solution from which "racemized" casein had been removed with ammonium sulphate an

¹ *This Journal*, xiii, p. 357, 1912.

albumose-like body, "racemized" caseose, was precipitated. The filtrate from this substance gave a precipitate with Hedin's tannic acid mixture,² as also the biuret test and tyrosine reaction with Millon's reagent. It therefore contained further simpler hydrolytic products.

The isolation of "racemized" casein and caseose from the digestion mixture is a point of some value. For in the work on gelatin no products were isolated but the mixtures were hydrolyzed as they stood. Thus, as was previously pointed out, the optical activity of certain of the amino-acids might be due simply to the fact that they were rapidly split off from the protein molecule by hydrolysis and so escaped racemization. This objection cannot be raised in connection with the amino-acids derived from "racemized" casein and caseose, and the occurrence of optically active amino-acids must be taken as evidence in favor of the view that they occupy terminal positions in the peptide chains.

The optical properties of corresponding amino-acids obtained by the hydrolysis of "racemized" casein and caseose were in every instance identical. Alanine was present in quantities so small that repeated recrystallization failed to secure a fraction of the pure substance. But a valine-alanine mixture rich in the latter was obtained, and this proved to be inactive in both aqueous and hydrochloric acid solutions. Thus inactive alanine was undoubtedly present. Whether any active alanine occurred could not be decided. Both *dextro* and inactive valine were isolated without much difficulty, and large quantities of *laevo* and inactive leucine were found. Phenylalanine, tyrosine, arginine, lysine, histidine, aspartic and glutamic acids were all inactive, while proline was active. An observation of some interest may be made here. The question as to the pre-existence of proline as such in the protein molecule has been raised by E. Fischer and often discussed. His experiments³ and those of Kossel⁴ on this subject have made it most probable that proline is a primary and not a secondary product. The fact that glutamic acid and arginine (ornithine) from "racemized" casein are optically inactive, while the proline is active, precludes the possibility of the latter being derived from

² *Journ. of Physiol.*, xxxii, p. 468.

³ *Zeitschr. f. physiol. Chem.*, xxxv, p. 227, 1902; *ibid.*, lxx, p. 118, 1910.

⁴ *Ibid.*, xl, p. 311, 1903.

the former during the process of hydrolysis, and is direct evidence in favor of the view that proline itself is a protein constituent. The activity of proline obtained from "racemized" gelatin, casein and caseose would suggest that the carboxyl group may be free. In this case the peptide linkage must occur with the nitrogen atom of the proline molecule, a conclusion which has been reached by Kossel and Gawrilow⁵ from investigations concerning the free amino-groups in proteins.

Of course the possibility of linkage by means of the carboxyl group is not absolutely precluded by our results, since in the communication on gelatin it was pointed out that prolylglycine is apparently not racemized by treatment with dilute alkali. Experiments with peptides of known structure are being carried out to elucidate this point.

The total inactivity of the bases, histidine, arginine and lysine may be regarded as evidence that they were attached to the protein molecule by condensation of their carboxyl groups with the amino groups of other constituents, results which indicate a similar linking to that of these bases in certain protamines investigated by Kossel and his collaborators.⁶

Both "racemized" casein and caseose gave the Adamkiewicz-Hopkins reaction for tryptophane, but attempts to isolate it failed, owing to the surprising fact that both substances were entirely resistant to the action of trypsin, and also of pepsin and erepsin.⁷

This absolute stability against the common proteolytic enzymes suggests that the racemization due to the tautomeric change $>\text{CH}-\text{CO}-\text{NH}- \rightleftharpoons >\text{C}=\text{C}.\text{OH}-\text{NH}-$ must be complete for every such group in the protein molecule. Otherwise, if a few groups ($>\text{CH}-\text{CO}-\text{NH}-$) retaining their natural configuration remained intact, one might expect enzymes to effect hydrolysis at such points yielding simpler peptones and peptides. So far no evidence of such partial splitting has been obtained.

The racemization of proteins at low temperatures by the action of dilute alkalies may in part account for the wide variation which

⁵ *Zeitschr. f. physiol. Chem.*, lxxxi, p. 274, 1912.

⁶ *Zeitschr. f. physiol. Chem.*, lxxii, p. 486, 1911; lxxviii, p. 402, 1912; lxxxiv, p. 1, 1913.

⁷ *This Journal*, xv, p. 271, 1913.

have been observed in the optical properties of tryptophane,⁸ and for the isolation of racemic tryptophane by Ellers.⁹

In conclusion, these results raise a doubt as to whether it will ever be possible by existing methods to synthesize a naturally occurring protein. For treatment of compounds containing peptide linkages with alkali occurs in all these processes, and with large molecules of this character racemization of the type described in this paper would most probably occur.

In view of these studies it would appear to be of importance to avoid the use of alkali in the extraction and purification of proteins. This procedure is often employed but must effect partial racemization and hence is objectionable. The difficulty of carrying out tryptic digestion until the biuret test becomes absolutely negative is a matter of common experience. This is in all probability due to partial racemization of the protein either occasioned by its mode of preparation or actually occurring during digestion. When complete tryptic digestion is wanted it is obviously necessary to keep the alkalinity of the solution as low as possible.

A comparative table showing the optical properties of the amino-acids derived from "racemized" gelatin, casein and caseose is here appended.

	GELATIN	CASEIN	CASEOSE
Alanine.....	<i>d</i> and inactive	inactive (and <i>d</i> ?)	inactive (and <i>d</i> ?)
Valine.....		<i>d</i> and inactive	<i>d</i> and inactive
Leucine.....	inactive	<i>l</i> and inactive	<i>l</i> and inactive
Tyrosine.....		inactive	inactive
Phenylalanine...	inactive	inactive	inactive
Proline.....	<i>l</i>	<i>l</i>	<i>l</i>
Aspartic acid....	inactive	inactive	inactive
Glutamic acid....	<i>d</i>	inactive	inactive
Arginine.....	inactive	inactive	inactive
Lysine.....	<i>d</i>	inactive	inactive
Histidine.....	inactive	inactive	inactive

These investigations are being continued.

⁸ Cf. *Zeitschr. f. physiol. Chem.*, lv, pp. 74 and 412, 1908.

⁹ *Biochem. Zeitschr.*, vi, p. 272, 1907.

EXPERIMENTAL.

Preparation of "racemized" casein and caseose.

500 grams of commercial casein are shaken up with 5 liters of $\frac{N}{2}$ sodium hydroxide until a homogenous mixture is obtained. After standing for a day in the incubator at 37°C. the liquid is filtered from the flocculent precipitate formed and the clear brown filtrate is returned to the incubator for 18-20 days when the rotation is found to have dropped to a constant value. The change in rotation of such a solution is from about -5° to -3° . A considerable amount of ammonia is evolved during incubation.

The brown liquid is then neutralized, while still warm, with sulphuric acid and becomes slightly turbid. Glacial acetic acid is added in small quantities at a time with constant stirring until no more precipitation of "racemized" casein occurs. The casein comes out in white flakes which adhere to the stirring rod, forming a plastic, dough-like mass which is best removed from the solution in this way. The small amount remaining in the liquid settles and adheres to the bottom of the vessel when the liquid may be poured off and the remainder of the "racemized" casein scraped out. The plastic substance is then pulled into small pieces which are dropped into water, which is subsequently poured off. The process is repeated until the wash water no longer shows an acid reaction to litmus. The racemized casein is then placed on porous plate and allowed to dry at room temperature for several days. It finally assumes a brown appearance and becomes friable. It may then be reduced to a fine white powder, resembling ordinary casein in appearance.

The average yield from 500 grams of casein is 100 grams of the "racemized" product.

Analysis of "racemized" casein.

The substance was dried in a steam oven.

0.1548 gram gave 0.3040 gram CO_2 and 0.0979 gram H_2O .

0.4940 gram required by Kjeldahl's method 16.75 cc. $\frac{N}{2}$ HCl whence

C = 53.55 per cent, H = 7.03 per cent, N = 12.5 per cent.

The acid liquid after removal of "racemized" casein is concentrated on the water bath and saturated with ammonium sulphate. A dark brown, sticky mass of "racemized" caseose is

precipitated, which, after purification from adhering salts and acetic acid, if spread in thin layers and exposed to the air for some time, becomes hard and brittle and may be ground in a mortar to a fine white powder. This substance is soluble to a practically unlimited extent in water, and exhibits more acidic properties than "racemized" casein.

A determination of the amount of complete hydrolysis brought about by the alkali was made by estimating the total nitrogen of the digestion liquid and comparing it with the nitrogen non-precipitable by phosphotungstic acid. A tenth of the total nitrogen was thus found to remain in solution.

Hydrolysis of "racemized" casein and caseose.

340 grams of dry "racemized" casein and 600 grams of moist, crude "racemized" caseose were hydrolyzed with hydrochloric acid for investigation of the amino-acids according to Fischer's ester method.

Alanine was obtained in a fraction together with valine which was inactive in both cases. Other mixtures whose analyses indicated the presence of some alanine were active, but this may have been due to the presence of *d*-valine.

Valine was obtained inactive in mixture with alanine, and also in mixtures of *r*- and *d*-valine containing over 50 per-cent of the latter variety.

Leucine was obtained pure in the inactive form, and also in mixtures of *l*- and *r*-leucine. Mixtures of leucine and valine which showed optical activity were also obtained.

Tyrosine was isolated by hydrolyzing the digestion mixture of 50 grams of casein as it stood with hydrochloric acid. The solution was evaporated *in vacuo*, the residue, dissolved in water, treated with animal charcoal and filtered. Ammonia was added, the solution concentrated and tyrosine allowed to crystallize out. The crude product was washed with hot glacial acetic acid, dissolved in hydrochloric acid and liberated by adding ammonia. It crystallized in characteristic form. It was found to be inactive in hydrochloric acid solution, a 25 per cent solution being examined in a 2.2 dm. tube. It follows that the tyrosine in both "racemized" casein and caseose is inactive.

Phenylalanine was obtained pure in large quantities and proved to be inactive in hydrochloric acid solution in both cases.

Proline. The rotation of the proline isolated from "racemized" casein indicated the presence of 48.9 per cent of the *laevo* form, while from "racemized" caseose a specimen was obtained which contained 30.7 per cent of the active variety. Since proline suffers partial racemization during the process of isolation it is probable that no racemization occurred before hydrolysis.

Aspartic and glutamic acids were obtained from both substances. The yields of the former were small, but both acids were completely inactive.

Arginine, lysine and histidine were isolated by the method of Kossel and Kutscher from 100 grams dry "racemized" casein and 125 grams crude "racemized" caseose respectively. The histidine, arginine, and lysine fractions were all optically inactive. The lysine fractions proving to be totally inactive rendered it unnecessary to isolate ornithine from them.

THE ACTION OF ENZYMES ON RACEMIZED PROTEINS, AND THEIR FATE IN THE ANIMAL BODY.

BY H. D. DAKIN AND H. W. DUDLEY.

(From the Herter Laboratory, New York City.)

(Received for publication, June 12, 1913.)

In this communication we are concerned with the behavior of "racemized" casein and caseose described in the preceding paper when subjected to the actions of pepsin, trypsin and erepsin. It was surprising to find that these simple derivatives of casein were both entirely resistant to the above enzymes, no hydrolysis taking place in any case with experiments *in vitro*. It was clearly of interest to determine what would be the fate of these substances in the animal body, since the results of such experiments might throw light upon the mechanism of intestinal absorption. On feeding to a dog by mouth, it was found that both substances were excreted unchanged in the feces, no absorption at all having taken place in the intestine.

It thus appears probable that the intestine is incapable of absorbing, as such, even substances of the complexity of albumoses, and can only take up bodies of simpler molecular structure.

It might be objected against this view that absorption might not take place with a body whose behavior towards enzymes is so unusual, while a substance of similar complexity, but normal (*i.e.*, digestible) to enzymes conceivably could be absorbed. But Dakin¹ has shown that certain simple optical enantiomorphs are equally well absorbed although enzymes may attack one stereoisomer much more readily than the other, so that the above argument seems improbable. It is more likely that intestinal absorption is a physical process, and it would be of great interest to ascertain the highest degree of molecular complexity that constituents of proteins and other bodies may possess and at the same time undergo absorption in the intestine. We shall attempt to obtain

¹ This *Journal*, iv, p. 437, 1903.

simpler fragments of the "racemized" protein molecule in the hope of further elucidating this question, *i.e.*, to what extent the protein molecule must be broken down before the fragments become capable of absorption in the intestine.

Given subcutaneously in concentrated aqueous solution to a dog, "racemized" caseose was excreted unchanged in the urine, the animal showing no symptoms.

The action of putrefactive bacteria was also tried on the two substances. The "racemized" casein remained unchanged, but the caseose was slowly attacked yielding indol and other products.

EXPERIMENTAL.

Methods. Solutions of the two substances were made under conditions favoring the action of the particular enzyme under investigation. Similar solutions of ordinary casein were prepared and used as controls, with pepsin and trypsin, and a solution of Witte's peptone was employed in the case of erepsin.

Equal amounts of the enzyme solution were then added to the three solutions, from which 5 cc. were immediately pipetted off and the total nitrogen estimated according to Kjeldahl. Estimations of the non-precipitable nitrogen were also immediately made by taking a known amount of the solutions, precipitating with a known volume of a suitable reagent, either Hedin's "tannic acid mixture"² or a 33 per cent solution of trichloroacetic acid, and then estimating the nitrogen in the filtrate by Kjeldahl's method, after the mixtures had stood for eighteen hours.

Periodically such non-precipitable nitrogen determinations were carried out, and thus it was possible to follow the course of the hydrolyses in the solutions, which were kept at a temperature of 37°C. in an incubator.

It will be noted that in the case of "racemized" caseose the non-precipitable nitrogen was comparatively high from the beginning of the experiment. This was largely caused by the presence of some ammonium sulphate derived from the salting-out process.

² *Journ. of Physiol.*, xxxii, p. 468.

Comparative hydrolyses of ordinary casein, "racemized" casein, and caseose by pepsin.

Four grams of each of the substances were dissolved in 100 cc. of $\frac{N}{10}$ HCl. To each of these solutions 0.1 gram of Merck's pepsin, dissolved in 5 cc. of water, was added together with a few drops of toluene. Total and non-precipitable nitrogen determinations were made immediately. Precipitation was effected with the ordinary casein and "racemized" casein by adding 5 cc. of a 33 per cent trichloroacetic acid solution to 10 cc. of the solutions. With the solution of "racemized" caseose this reagent gave only a turbid, milky emulsion, so that in this case 10 cc. of the solution were precipitated with 10 cc. of tannic acid mixture. After standing for eighteen hours the non-precipitable nitrogen was determined in the filtrate. The solutions were kept at 37°C. and periodically non-precipitable nitrogen determinations were made. The results are given in the following table.

SUBSTANCE	TOTAL NITROGEN PER 100 cc.	NON-PRECIPITABLE NITROGEN PER 100 cc.		
		0 hours	24 hours	43 hours
	gram	gram	gram	gram
Casein.....	0.434	0.042	0.245	0.294
"Racemized" casein.....	0.406	0.042	0.063	0.063
"Racemized" caseose.....	0.497	0.259	0.259	0.259

Comparative hydrolyses of ordinary casein, "racemized" casein and caseose by trypsin.

Ten grams of ordinary casein and 10 grams of "racemized" casein were dissolved in 200 cc. of 0.8 per cent Na_2CO_3 solution. Ten grams of "racemized" caseose were dissolved in 200 cc. of water containing 1.6 grams of Na_2CO_3 . To each of these solutions were added 5 cc. of a trypsin solution prepared from ox pancreas by Hedin's method³ and 2 cc. of toluene. Total nitrogen and non-precipitable nitrogen determinations were made as in the above experiment, except that equal volumes of the solutions

³ Journ. of Physiol., xxxii, p. 468.

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and of tannic acid mixture (10 cc.) were used for the estimation of non-precipitable nitrogen in all cases. The digestions were carried out at 37°C. The results are as follows:

SUBSTANCE	TOTAL NITROGEN PER 100 cc.	NON-PRECIPITABLE NITROGEN PER 100 cc.			
		0 hours	24 hours	48 hours	72 hours
	gram	gram	gram	gram	gram
Casein.....	0.595	0.025	0.329	0.399	0.441
"Racemized" casein...	0.560	0.063	0.069	0.070	0.070
"Racemized" caseose..	0.504	0.217	0.245	0.245	0.245

As a confirmatory experiment 100 cc. of each of the above solutions of "racemized" casein and caseose were taken and 25 cc. of the trypsin solution were added. The nitrogen determinations were as follows:

SUBSTANCE	TOTAL NITROGEN PER 100 cc.	NON-PRECIPITABLE NITROGEN PER 100 cc.	
		0 hours	24 hours
	gram	gram	gram
"Racemized" casein.....	0.511	0.154	0.168
"Racemized" caseose.....	0.490	0.273	0.287

The slight rise in non-precipitable nitrogen was undoubtedly due to autolysis of the added enzyme solution.

Comparative hydrolyses of Witte's peptone, "racemized" casein and caseose by erepsin.

A solution of erepsin was prepared by digesting the mucosa of a dog's intestine at room temperature for fifteen hours with physiological saline solution, filtering through muslin, and employing the extract so obtained without further purification. A determination of the amount of autolysis in this solution showed that the non-precipitable nitrogen rose from 0.119 to 0.290 gram per 100 cc. liquid in forty-eight hours at 37°C. Five grams of Witte's peptone were dissolved in 100 cc. of water. The solution was faintly alkaline to litmus. 2.5 grams of racemized casein were dissolved to a slightly turbid solution in 100 cc. of 0.2 per cent Na_2CO_3 solution; the reaction to litmus was neutral. Five grams

of "racemized" caseose were dissolved up in 100 cc. of water. This solution was acid to litmus. Sodium carbonate solution was added until the alkalinity of the liquid was of the same order as that of the peptone solution.

To each of these solutions were added 10 cc. of the erepsin extract and 1 cc. of toluene. The usual nitrogen determinations were made, tannic acid mixture being used as precipitant. The results were as follows:

SUBSTANCE	TOTAL NITROGEN PER 100 cc.	NON-PRECIPITABLE NITROGEN PER 100 cc.		
		0 hours	24 hours	48 hours
	gram	gram	gram	gram
Witte's peptone.....	0.714	0.189	0.567	0.672
"Racemized" casein.....	0.280	0.023	0.046	0.065
"Racemized" caseose.....	0.483	0.168	0.196	0.210

The slight rises in the non-precipitable nitrogen of the "racemized" casein and caseose solutions are accounted for by the autolysis of the added erepsin extract.

Fate of "racemized" casein and caseose in the animal body.

Preliminary experiments showed that on giving small quantities of the two substances mixed with food to a dog, tests for proteins in the urine were negative.

The dog, after fasting, was put on a fixed diet of 100 grams of boiled rice and 20 grams of bone ash. At suitable intervals "racemized" casein was fed to the animal and the nitrogen elimination followed in twenty-four-hour periods. A comparative feeding of ordinary casein was made at the end of the experiment. The following table gives the results and indicates no absorption of nitrogen from the "racemized" protein.

PERIOD	TOTAL URINARY NITROGEN	SUBSTANCE FED	NITROGEN OF SUBSTANCE FED
	grams		grams
I	1.64	—	—
II	1.34	20 grams "racemized" casein	2.6
III	1.75	20 grams "racemized" casein	2.6
IV	2.31	20 grams ordinary casein	3.0

The feces passed after feeding "racemized" casein were extracted with 3 per cent sodium carbonate solution. The filtrate gave a strong biuret reaction and on acidifying "racemized" casein was precipitated.

Similar experiments with "racemized" caseose showed that it too passes through the gut without absorption and is readily recovered from the feces.

About 5 cc. of a concentrated aqueous solution of "racemized" caseose were injected subcutaneously into a dog. The animal showed no symptoms. After five hours urine was passed which gave a strong biuret reaction and a white turbidity with trichloroacetic acid, proving the presence of caseose. This urine gave no precipitate on boiling, nor on acidifying with acetic acid. A sample of urine collected before the injection was perfectly normal, and such was again the case a day after.

Action of putrefactive bacteria on "racemized" casein and caseose.

Two grams of "racemized" casein and of "racemized" caseose were dissolved in 20 cc. of 0.8 per cent sodium carbonate solution containing traces of calcium chloride, magnesium sulphate and sodium phosphate.

The solutions were inoculated with small amounts of putrid pancreas infusion. After ten days the "racemized" casein was unaffected while in the caseose solution the organisms had slowly grown, decomposing the caseose with formation of indol and other products of putrefaction.

STUDIES IN BACTERIAL METABOLISM. XI.

DETERMINATION OF "UREA NITROGEN" IN CULTURES OF CERTAIN BACTERIA.

BY ARTHUR I. KENDALL AND ARTHUR W. WALKER.

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(Received for publication, June 17, 1913.)

The qualitative and quantitative measure of nitrogen degradation is one of the fundamental steps in studying cellular metabolism, for nitrogen is one of the most important structural elements which enter into the composition of the cell. In man, nitrogenous waste leaves the body largely in the form of urea, urea comprising about 80 per cent of the total nitrogen excreted. Most of this urea is formed in a special organ, the liver, although it is almost certain that muscles and perhaps other tissues form a small amount besides. The question naturally presents itself—do other unicellular organisms form urea, or is the ultimate degradation of nitrogen in such organisms largely ammonia. If it could be shown, for example, that unicellular organisms such as bacteria form urea, the bearing of such observations upon multicellular organisms is obvious. With the bacteria, which appear to excrete the greater part of their waste nitrogen as ammonia, urea formation would in reality be a product of intermediary metabolism, inasmuch as it represents nitrogen in a more complex state of organization than ammonia. Many bacteria can actually utilize urea as a source of nitrogen.

In order to throw some light upon this possibility—that of urea formation—a considerable number of bacteria representing various degrees of proteolytic activity have been grown in sugar-free and dextrose-containing standard nutrient broth made from meat juice and peptone (Witte's). This broth has been examined after inoculation with these bacteria at stated intervals, using the new

Folin¹ urea method, for the presence of "urea nitrogen," comparing the results obtained with suitable controls. According to Folin the presence of dextrose interferes with this determination until definite dilutions of the sugar have been reached. Observations on the uninoculated plain and dextrose broth controls indicate that the decrease of "urea nitrogen" in sugar broth containing 1 per cent of dextrose is but about 0.70 mgm. per 100 cc. less than that of the corresponding sugar-free broth in our media. What effect the salts of the organic acids may have had upon the determinations is not definitely known, but it is safe to say that the results are comparable, because the content of these substances is the same in both kinds of media. The rather striking uniformity of the results obtained in dextrose broth makes them of comparative interest, if not of relative value.

The method, briefly, is as follows: (1) 7 grams of pure, dry, ammonia-free potassium acetate are placed in a large test tube (8 inches \times 1 inch), 1 cc. of 50 per cent acetic acid added, and 1 cc. of culture. The tube is closed with a rubber stopper containing a tube about 25 cm. long and 1 cm. in diameter. The mixture is heated to 155°C. for 20 minutes in a sulphuric acid bath, the condenser and stopper then washed down with a small amount of water, the solution made alkaline with 1 cc. of strong caustic soda and the ammonia blown into 10 cc. of $\frac{N}{50}$ HCl by an air current. At the same time the free ammonia is determined in another portion of this broth in the same manner in uninoculated controls. Determinations are made in duplicate: the greatest difference between these duplicate determinations was 0.1 cc. $\frac{N}{50}$ alkali, or 2.8 milligrams per 100 cc. of culture.

It is a fact that the "urea nitrogen" both in uninoculated media and in cultures of bacteria is far less than in urine, hence the percentage of error in these determinations is far greater even allowing for the very dilute standard solution used— $\frac{N}{50}$ HCl and NaOH respectively—than in corresponding urine determinations. The maximum error of the method as applied to bacterial cultures between duplicate determinations is roughly 2 per cent for the "urea nitrogen" and 1 per cent for the free ammonia determinations, hence the maximum total error is about 3 per cent.

¹Folin: this *Journal*, xi, pp. 507-522, 1912.

Considering the very small amounts of nitrogen involved, this error cannot be regarded as excessive: it is the best that can be accomplished with the methods available at the present time.

The results are expressed respectively as milligrams of "urea" and ammonia nitrogen per 100 cc. of broth: the difference between the "urea" nitrogen and the ammonia nitrogen, after subtracting the corresponding controls (uninoculated broth), indicates the gain or loss in "urea" nitrogen by the various bacteria studied.

The tables show that the "urea" nitrogen of the cultures increases proportionately to the ammonia nitrogen, and to about the same relative degree: generally speaking the initial proportional excess of "urea" nitrogen over ammonia nitrogen is neither increased nor decreased noticeably. This would appear to indicate that products of intermediary metabolism of the nitrogen-containing constituents of the broth which can be reduced to ammonia by heating to 155°C. with the reagents used are not present in noteworthy amounts. Ammonia formation, the final step in the degradation of protein and protein derivatives by the commonly met with bacteria, is the best available index of proteolysis by bacteria.

The organisms studied are fairly representative proteolytically, hence the results shown in the tables cover the possibilities of this determination for bacteria in a moderately complete manner. It cannot be stated definitely that the observed increase in "urea" nitrogen is referable to urea. The very small amount of reacting substances would make their isolation very difficult. It is very probable that uninoculated broth contains at least some urea, derived from the meat which is a basis for the media. Whether this urea originally present is decomposed by bacterial growth and is replaced by other nitrogenous products of proteolytic origin, or whether it persists as urea cannot be stated. Even in the urine, where urea is present in considerable amounts, it is by no means definitely settled that the determination of "urea" nitrogen represents urea alone.

ORGANISM	DAY OF OBSERVATION	PLAIN BROTH					DEXTROSE BROTH				
		"Urea" N	Increase in "Urea" N	NH ₄ -N	Increase in NH ₃ N	± "Urea" N	"Urea" N	Increase in "Urea" N	NH ₄ -N	Increase in NH ₃ N	± "Urea" N
Control		14.70		8.40		+6.30	13.30		8.40		+4.90
Grass bacillus	1	15.75	1.05	8.40	0.00	+1.05	13.30	0.00	8.40	0.00	0.00
	3	17.65	3.15	9.80	1.40	+1.75	14.00	0.70	7.00	-1.40	+2.10
	6	17.50	2.80	11.20	2.80	0.00	14.70	1.40	9.80	1.40	0.00
	9	21.00	6.30	15.40	7.00	-0.70	18.90	5.60	9.10	0.70	+4.90
B. coli I	1	18.20	3.50	10.50	2.10	+1.40	14.70	1.40	8.40	0.00	+1.40
	3	22.40	7.70	16.10	7.70	0.00	14.00	0.70	8.75	0.35	+0.35
	6	27.30	12.60	18.90	10.50	+2.10	16.80	3.50	8.75	0.35	+3.15
	9	30.10	15.40	20.30	11.90	+3.50	16.80	3.50	8.75	0.35	+3.15
B. proteus II	1	16.80	2.10	9.80	1.40	+0.70	15.40	2.10	8.40	0.00	+2.10
	3	21.70	7.00	14.70	6.30	+0.70	15.40	2.10	9.10	0.70	+1.40
	6	33.60	18.90	25.90	17.50	+1.40	17.50	4.20	9.10	0.70	+3.50
	9	41.30	26.60	36.40	28.00	-1.40	18.20	4.90	9.10	0.70	+4.20
Sp. Finkler and Prior	1	16.80	2.10	9.10	0.70	+1.40	14.00	0.70	8.40	0.00	+0.70
	3	22.40	7.70	14.00	5.60	+2.10	15.40	2.10	8.40	0.00	+2.10
	6	26.60	11.90	18.90	10.50	+1.40	15.40	2.10	9.10	0.70	+1.40
	9	28.70	14.00	21.70	13.30	+0.70	16.80	3.50	9.10	0.70	+2.80
B. cloacae	1	16.10	1.40	9.10	0.70	+0.70	16.10	2.80	7.70	-0.70	+3.50
	3	18.20	3.50	13.30	4.90	-1.40	16.80	3.50	9.10	0.70	+4.20
	6	24.50	9.80	16.80	8.40	+1.40	19.60	6.30	11.90	3.50	+2.80
	9	26.60	11.90	18.50	10.10	+1.80	35.00	21.70	17.50	9.10	+12.60

Mic. zymogenes	1	16.10	1.40	8.80	0.40	+1.00	15.40	2.10	9.80	1.40	+0.70
	3	16.80	2.10	8.80	0.40	+1.70	15.40	2.10	8.40	0.00	+2.10
	6	16.80	2.10	8.80	0.40	+1.70	16.80	3.50	8.75	0.35	+3.15
	9	16.80	2.10	9.10	0.70	+1.40	17.50	4.20	8.75	0.35	+3.85
B. dysenteriae Flexner,	1		2.10	9.10	0.70	+1.40	13.30	0.00	8.40	0.00	0.00
	3	16.80	2.10	0.10	0.70	+1.40	16.80	3.50	8.40	0.00	+3.50
	6	16.80	2.80	10.15	1.75	+1.05	16.80	3.50	0.10	-0.70	+2.80
	9	17.50				<td></td> <td></td> <td></td> <td></td> <td></td>					
Control		14.00		7.35		+0.65	13.30		7.00		+0.30
Hog cholera I (avirulent)	1	14.70	0.70	7.75	0.35	+0.35	13.30	0.00	7.00	0.00	0.00
	3	17.50	3.50	11.55	4.20	-0.70	13.30	0.00	7.70	0.70	-0.70
	6	22.40	8.40	19.25	11.00	-3.50	14.00	0.70	7.70	0.70	0.00
	9	23.10	9.10	10.25	11.90	-2.80	14.00	0.70	7.70	0.70	0.00
Hog cholera II (virulent)	1	14.70	0.70	7.70	0.35	+0.35	13.30	0.00	7.35	0.35	-0.35
	3	16.80	2.80	10.50	3.15	-0.35	12.00	-0.70	7.70	0.70	-1.40
	6	18.20	4.20	11.20	3.85	+0.35	13.30	0.00	7.70	0.70	-0.70
	9	18.20	4.20	11.00	4.55	-0.35	14.00	0.70	7.70	0.70	0.00
B. typhosus	1	14.00	0.00	7.35	0.00	0.00	13.30	0.00	7.00	0.00	0.00
	3	14.00	0.00	0.80	2.45	-2.45	13.30	0.00	7.35	0.35	-0.35
	6	15.40	1.40	9.80	2.45	-2.45	13.30	0.00	7.70	0.70	-0.70
	9	18.20	4.20	10.85	3.50	+0.70	14.00	0.70	7.70	0.70	-0.00
Morgan bacillus	1	21.00	7.00	14.70	7.35	-0.35	14.00	0.70	8.05	1.05	-0.35
	3	21.70	7.70	15.75	8.40	-0.70	14.00	0.70	8.75	1.75	-1.05
	6	21.70	7.70	17.50	10.15	-2.45	14.00	0.70	8.75	1.75	-1.05
	9	22.40	8.40	17.50	10.15	-1.75	14.00	0.70	9.10	2.10	-1.40

ORGANISM	DAY OF OBSERVATION	PLAIN BROTH					DEXTROSE BROTH				
		"Urea" N	Increase in "Urea" N	NH ₃ -N	Increase in NH ₃ N	± "Urea" N	"Urea" N	Increase in "Urea" N	NH ₃ -N	Increase in NH ₃ N	± "Urea" N
Control		14.00		7.35		6.65	13.30		7.00		6.30
Paratyphoid I	9	16.80	2.80	9.80	2.45	+0.35	13.30	0.00	7.70	0.70	-0.70
Paratyphoid II	9	16.80	2.80	10.15	2.80	0.00	13.30	0.00	7.70	0.70	-0.70
Paratyphoid III	9	16.80	2.80	10.15	2.80	0.00	13.30	0.00	7.70	0.70	-0.70
Paratyphoid IV	9	16.10	2.10	9.80	2.45	-0.35	13.30	0.00	7.70	0.70	-0.70
Paratyphoid V	9	16.80	2.80	10.15	2.80	0.00	12.60	-0.70	8.05	1.05	-1.75
Paratyphoid VI	9	16.80	2.80	10.15	2.80	0.00	11.90	-1.40	7.35	0.35	-1.75
Paratyphoid alpha I	9	17.50	3.50	14.00	6.65	-3.15	13.30	0.00	7.70	0.70	-0.70
Paratyphoid alpha II	9	16.10	2.10	9.80	2.45	-0.35	12.60	-0.70	7.70	0.70	-1.40
Paratyphoid alpha III	9	16.80	2.80	13.30	5.95	-3.15	13.30	0.00	7.70	0.70	-0.70
Morgan bacillus I	9	22.40	8.40	18.20	10.85	-2.45	13.30	0.00	8.05	1.05	-1.05
Morgan bacillus II	9	21.00	7.00	17.50	10.15	-3.15	13.30	0.00	9.10	2.10	-2.10
Morgan bacillus III	9	22.40	8.40	18.20	10.85	-2.45	13.30	0.00	9.10	2.10	-2.10
Morgan bacillus IV	9	21.70	7.70	17.50	10.15	-2.45	13.30	0.00	8.05	1.05	-1.05
Hog cholera III	9	16.80	2.80	10.15	2.80	0.00	12.60	-0.70	7.70	0.70	-1.40
Hog cholera IV	9	21.00	7.00	15.40	8.15	-1.05	13.30	0.00	7.35	0.35	-0.35
Fowl cholera I	9	18.90	4.90	13.30	5.95	-1.05	12.60	-0.70	7.70	0.70	-1.40
B. acidilactici II	9	17.50	3.50	12.60	5.25	-1.75	13.30	0.00	7.84	0.84	-0.84
B. acidilactici III	9	17.50	3.50	11.55	4.20	-0.70	13.30	0.00	7.84	0.84	-0.84
Swine plague	9	17.50	3.50	9.45	2.10	+1.40	12.60	-0.70	7.70	0.70	-1.40
Shiga bacillus	9	16.10	2.10	9.45	2.10	0.00	12.60	-0.70	7.70	0.70	-1.40
Cholera IV	9	33.60	19.60	26.95	19.60	0.00	12.60	-0.70	7.70	0.70	-1.40
Diphtheria VIII	9	15.40	1.40	9.45	2.10	-0.70	12.60	-0.70	7.00	0.00	-0.70

THE INFLUENCE OF STARVATION UPON THE CREATINE CONTENT OF MUSCLE.

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Considerable interest has been attached to the metabolism of creatine since Benedict¹ in 1907 noted its appearance in the urine in a series of experiments upon starving men, and subsequently, in collaboration with Diefendorf,² confirmed this observation upon a starving woman. This excretion of creatine in starving men was likewise noted independently by Cathcart,³ while the experiments of Dörner,⁴ and Mendel and Rose⁵ on rabbits, and the observations of Underhill and Kleiner,⁶ Richards and Wallace,⁷ and Howe and Hawk⁸ upon starving dogs would indicate that the excretion of creatine is common to starving mammals. It should be noted, however, that McCollum and Steenbock⁹ have shown that in the pig inanition does not readily cause an elimination of creatine, which is in striking contrast to the ease with which its excretion is produced in the rabbit.

That creatine may appear in the urine in a variety of pathological conditions, especially in those associated with a loss in weight and under-nutrition, was pointed out by Benedict and one

¹ Benedict, F. G.: Carnegie Inst., Washington, Publ. No. 77, p. 386, 1907.

² Benedict and Diefendorf: *Amer. Journ. of Physiol.*, xviii, p. 362, 1907.

³ Cathcart: *Journ. of Physiol.*, xxxv, p. 500, 1907.

⁴ Dörner: *Zeitschr. f. physiol. Chem.*, lii, p. 225, 1907.

⁵ Mendel and Rose: *this Journal*, x, p. 213, 1911. A discussion of the literature is given in this paper.

⁶ Underhill and Kleiner: *ibid.*, iv, p. 165, 1908.

⁷ Richards and Wallace: *ibid.*, iv, p. 179, 1908.

⁸ Howe and Hawk: *Journ. of Amer. Chem. Soc.*, xxxiii, p. 215, 1911; also Howe, Mattill and Hawk: *this Journal*, x, p. 417, 1911.

⁹ McCollum and Steenbock: *ibid.*, xiii, p. 209, 1912.

of us,¹⁰ and subsequently confirmed by many workers. The more important conditions in which an elimination of creatine has been detected are carcinoma of the liver,¹¹ exophthalmic goitre,¹² muscular dystrophy,¹³ anterior poliomyelitis,¹⁴ typhoid fever,¹⁵ pneumonia,¹⁶ diabetes mellitus,¹⁷ pernicious vomiting of pregnancy,¹⁸ and following pregnancy. The elimination of creatine during the post partum resolution of the uterus in women was first noted by Shaffer,¹⁹ and subsequently observed in dogs by Murlin.²⁰ In a recent communication, Mellanby²¹ puts an entirely new interpretation upon this excretion of creatine. He has observed that the elimination is coincident with the secretion of milk, the excretion being of the same intensity where the uterus was removed in a case of Caesarian section.

As might be inferred from the foregoing observations, creatine is not normally a constituent of the urine in the adult, although Rose²² has recently shown that it is constantly present in the urine of children, an observation which has been confirmed by Folin and Denis.²³ Just why children eliminate creatine is difficult to explain. In a still more recent communication, McCollum and Steenbock²⁴ report the excretion of creatine in growing pigs when on a high protein intake from certain sources. Since an equally high protein intake from another source may be without this

¹⁰ Benedict and Myers: *Amer. Journ. of Physiol.*, xviii, p. 406, 1907.

¹¹ Mellanby: *Journ. of Physiol.*, xxxvi, p. 447, 1908; van Hoogenhuyze and Verploegh: *Zeitschr. f. physiol. Chem.*, lvii, p. 161, 1908.

¹² Shaffer: *Amer. Journ. of Physiol.*, xxiii, p. 1, 1908.

¹³ Levene and Kristeller: *ibid.*, xxiv, p. 45, 1909.

¹⁴ *Ibid.*

¹⁵ Klercker: *Zeitschr. f. klin. Med.*, lxxviii, p. 22, 1909; Ewing and Wolf: *Arch. of Int. Med.*, iv, p. 330, 1909; Shaffer and Coleman: *ibid.*, iv, p. 538, 1909.

¹⁶ Klercker: *loc. cit.*; Wolf and Lambert: *ibid.*, v, p. 406, 1910.

¹⁷ Shaffer: *loc. cit.*; Krause: *Quart. Journ. of Exp. Physiol.*, iii, p. 289, 1910; Taylor: *Biochem. Journ.*, v, p. 362, 1911.

¹⁸ Underhill and Rand: *Arch. of Int. Med.*, v, p. 61, 1910.

¹⁹ Shaffer: *loc. cit.*

²⁰ Murlin: *Amer. Journ. of Physiol.*, xxviii, p. 422, 1911.

²¹ Mellanby: *Proc. Roy. Soc., B*, lxxxvi, p. 88, 1913.

²² Rose: this *Journal*, x, p. 265, 1911.

²³ Folin and Denis: *ibid.*, xi, p. 253, 1912.

²⁴ *Loc. cit.*

effect, they consider this a demonstration of an exogenous excretion of creatine.

That the creatine eliminated in the urine during starvation was derived from the creatine of the muscle, was the original supposition of Benedict,²⁵ though few data have been submitted definitely bearing on this point. Mendel and Rose²⁶ have recently investigated the influence of starvation upon the creatine content of muscle. They have observed a very appreciable increase in the creatine concentration of muscle during starvation in seven out of eight experiments upon rabbits, and four out of five experiments upon hens. From these observations they conclude that without doubt there occurs an increase in the percentage of creatine in the muscles of rabbits and hens during inanition. This they interpret as due to an increased creatine formation. Aside from these observations, Dorner²⁷ has reported one experiment upon a starving rabbit in which a decreased concentration of creatine was observed, while Howe and Hawk²⁸ have reported an experiment upon a starving dog in which a marked reduction in the creatine content of the muscle was found. Further discussion of this subject will be made in connection with our experiments.

In an earlier communication²⁹ attention was called to the constancy in the content of muscle creatine for normal animals of a given species, though distinctive for different animals, *e.g.*, 0.52 per cent for the rabbit, 0.37 per cent for the dog, etc. In the case of the rabbit, observations upon the creatine concentration of the muscle were reported for twenty animals, the results being very uniform and many of them identical. It was suggested that this might serve to explain the constancy in the elimination of creatinine. In twelve experiments determinations were also made of the total amount of creatine present in the tissues of the body, and the ratio which existed between this and the amount of the daily elimination of creatinine. For animals of nearly the same weight, the ratio was very constant. It is reasonable to believe that if the creatine eliminated in the urine during starvation

²⁵ *Loc. cit.*

²⁶ Mendel and Rose: *this Journal*, x, p. 255, 1911.

²⁷ *Loc. cit.*

²⁸ *Loc. cit.*

²⁹ Myers and Fine: *this Journal*, xiv, p. 9, 1913.

owes its origin to the disintegration of muscle tissue, the total creatine of the body will be depleted in proportion to the amount eliminated in the urine, provided creatine is not readily destroyed in the body. It was upon this hypothesis that the present investigation was undertaken; in fact, the data previously reported were in part carried out as a control to those reported in the present paper.

METHODS EMPLOYED.

In general, the methods employed were the same as those described in our previous paper. The present experiments were made upon twenty-two rabbits, eighteen of which were subjected to single periods of starvation, while the remaining four were deprived of food for two ten-day periods, a feeding period of ten days being interposed between these two periods. In all cases a control period of one week was obtained, during which the animals were fed upon a uniform diet of 350-400 grams of carrots. After the control period in the first series of experiments, the animals were starved to the point of death. The urines were collected in weekly periods, the daily samples being preserved with toluene at 0°C. during the interval. The analytical determinations in these periods included total nitrogen, creatinine and creatine. In the majority of cases the animals were killed when it was certain they would not survive more than a few hours longer. In some cases, however, the animals died unexpectedly during the night, in which event the samples of muscle and the carcass were ground up and covered with alcohol as soon as possible on the following morning. It is not believed that any very appreciable error was introduced in this way.³⁰ The carcass and

³⁰ Several experiments were made to secure light on this point. Four may be cited. A sample of muscle from rabbit 64 had a moisture content of 73.8 per cent and a creatine content of 0.534 per cent. Upon allowing a weighed sample of muscle to stand at room temperature without any preservative for two days the same content of creatine was found. A similar experiment was performed with rabbit 65 allowing the sample to stand three days with no change in the content of creatine (total creatinine). Rabbit 63 was allowed to remain at room temperature for twenty-four hours before taking the samples of muscle. The muscle was then found to have a moisture content of 76.7 per cent, a nitrogen content of 3.28 per cent, while two separate samples were both found to contain 0.516 per cent

a sample of muscle from the hind legs were analyzed for creatine as previously described. In many cases it was not possible to obtain 100 gram samples as was done with the normal animals, and in these instances 40 grams were generally taken. In addition, moisture and nitrogen determinations were made on the muscle in most cases.

The rabbit is a particularly suitable animal for the experiments described, by reason of the ease with which starvation causes an excretion of creatine, and further, because the size of the animal makes a determination of the total creatine content of the body comparatively easy.

On account of the very large amount of data, the detailed protocols are not included in this paper. The more important facts in the first series of experiments may be found in Table I, those of the second series in Table IX. Special tables have been made for the individual topics of interest.

EXPERIMENTAL PART.³¹

In the first series of eighteen rabbits with a single period of starvation, the length of life, as shown in Table I, varied between six and twenty-seven days, with an average of two weeks. The loss in weight varied from 35 to 50 per cent, depending upon the length of the fast.

Influence of starvation upon the content of muscle creatine.

As may be observed in Table I, the content of creatine in the muscle is relatively increased in a short fast, while in a long fast it is generally decreased. This decrease is, furthermore, often accentuated by the high content of moisture (about 80 per cent).

creatine. In the case of rabbit 6S, an analysis of the muscle of the right leg, in which samples were taken at once after killing, yielded: moisture, 78.4 per cent, nitrogen 3.30 per cent and creatine 0.522 per cent. Twenty-four hours later, samples of muscle were removed from the other leg with the following result: moisture, 79.4 per cent, nitrogen 3.05 per cent and creatine 0.511 per cent.

³¹ A preliminary report of these experiments was made to the Society for Experimental Biology and Medicine, October 16, 1912, see *Proceedings*, x, p. 12, 1912. We were assisted in these experiments by Mr. Adolph Bernhard.

TABLE I.
Influence of starvation upon the creatine content of the body—summary table.

ANIMAL	LENGTH OF STARVATION days	Body weight			Composition of muscle			Creatine content of body tissues, etc.				
		Initial weight	Weight at death	Loss in weight	Molature	Nitrogen	Creatine	Creatine excreted in urine	Creatine content of tissues at death	Creatine content of body at death	Creatine of tissues and urine	Creatine of tissues and urine in terms of initial weight
		kgrs.	kgrs.	per cent	per cent	per cent	per cent	grams	grams	per cent	grams	per cent
37	6	1.55	0.98	36.8	78.3	3.47	0.550	0.73	1.71	0.174	2.44	0.157
41*	7	2.34	1.83	21.8	75.6	3.91	0.573	0.22	3.87	0.211	4.09	0.175
17	8	1.91	1.21	36.7			0.587	0.79	2.61	0.215	3.40	0.170
13†	9	1.97	1.26	36.0			0.618	0.58	2.60	0.206	3.18	0.159
44	10	1.85	1.06	42.7	79.4	3.38	0.524	1.28	1.69	0.160	2.98	0.161
24†	11	1.64	0.91	44.5	80.2	3.22	0.380	0.91	1.27	0.140	2.18	0.133
40	12	1.84	1.04	43.5	79.5	3.21	0.417	2.07				
20†	13	1.77	1.07	39.6	76.0		0.556	0.62	2.19	0.205	2.81	0.159
21†	14	1.63	0.93	42.0			0.313	1.26	1.17	0.126	2.43	0.149
23	14	1.46	0.83	43.1	81.1		0.397	1.30	1.26	0.152	2.50	0.171
38	15	1.78	1.12	37.1	78.6	3.53	0.492	0.90	1.88	0.167	2.77	0.156
19†	15	1.81	1.09	39.7			0.397	1.24	1.70	0.156	2.95	0.163
43	17	1.74	0.94	46.0	77.6	3.64	0.426	1.51	1.17	0.125	2.68	0.154
42†	19	1.88	1.01	46.3	80.3	3.24	0.357	1.20	1.28	0.126	2.48	0.132
36†	22	2.79	0.95	46.9	79.1	3.30	0.402	1.55	1.51	0.158	3.06	0.171
39	24	1.27	1.10	51.6	80.8	3.19	0.361	1.91	1.34	0.122	3.25	0.143
14†	25	1.69	0.89	47.3			0.522	0.49	1.91	0.215	2.40	0.140
16††	27	2.33	1.19	48.9	77.6	3.70	0.382	0.89	1.91	0.161	2.80	0.120

* Rabbit killed at the end of one week, though it obviously would have lived longer.

† Animal died.

†† Rabbits 14 and 16 both pregnant and aborted early in starvation, their power of resistance apparently increased.

TABLE I—CONCLUDED.

ANIMAL	CREATININE ELIMINATED IN URINE DURING STAR- VATION IN TERMS OF CRE- ATININE		CREATININE OF TISSUES AND OF URINE AS CREATININE IN TERMS OF INITIAL WT.		AVERAGE DAILY LOSS OF			LOSS PER KG. OF LOSS IN WEIGHT			IN PERCENTAGE OF TOTAL NITROGEN AVERAGE FIGURES				AVERAGE DAILY ELIMINATION OF CREATININE		
	grams	per cent	Weight	Nitrogen	Creatinine	Nitrogen	Creatinine N	Creatinine and cre-	grams	Creatinine N	Creatinine and cre-	Creatinine N	Creatinine N	Creatinine and cre-	mgms.	mgms.	per cent
37	0.35	0.171	95	1.31	122	16.1	0.41	0.61	2.6	1.2	3.8	3.8	40	55	-11		
41	0.72	0.205	73	0.86	32	11.8	0.14	0.59	1.2	3.8	5.0	5.0	88	86	+2		
17	0.55	0.197	88	1.19	99	13.7	0.36	0.61	2.7	1.8	4.5	4.5	59	71	-17		
13	0.55	0.186	70	1.16	65	14.7	0.26	0.50	1.8	1.6	3.4	3.4	51	69	-26		
44	0.68	0.108	79	1.40	128	18.9	0.52	1.01	2.8	1.5	4.3	4.3	59	62	-5		
21	0.66	0.103	66	1.33	83	20.0	0.40	0.69	2.0	1.4	3.4	3.4	51	66	-22		
40	0.93		68	1.32	124	19.7	0.60	0.97	3.0	1.9	4.9	4.9	67	63	+0		
20	0.71	0.100	82	0.80	47	14.9	0.28	0.61	1.9	2.2	4.1	4.1	47	74	-36		
21	0.86	0.201	50	0.91	90	18.1	0.58	0.77	3.2	2.2	5.4	5.4	53	77	-31		
23	0.69	0.219	41	1.17	93	26.0	0.66	1.05	2.6	1.4	4.0	4.0	42	58	-28		
38	1.26	0.224	41	1.00	60	18.0	0.44	1.05	1.9	2.7	4.6	4.6	72	49	+47		
19	1.01	0.221	73	1.09	83	22.8	0.55	1.02	2.4	2.0	4.4	4.4	60	76	-21		
43	1.18	0.222	47	1.15	63	24.5	0.60	1.08	2.5	1.9	4.4	4.4	60	66	-9		
42	1.44	0.209	46	1.01	63	22.0	0.44	0.97	2.0	2.4	4.4	4.4	66	73	-10		
36	1.33	0.245	38	0.96	71	25.2	0.59	1.11	2.4	2.0	4.4	4.4	52	65	-20		
39	1.97	0.230	49	1.01	80	21.3	0.52	1.06	2.5	2.5	5.0	5.0	71	81	-16		
14	1.43	0.221	32	0.49	18	15.3	0.18	0.71	1.2	3.4	4.6	4.6	45	61	-26		
16	1.70	0.107	42	0.60	33	16.4	0.25	0.75	1.5	3.1	4.6	4.6	57	88	-35		

The influence of starvation upon the content of muscle creatine is best shown in Table II, in which the data have been arranged in the order of creatine concentration.

TABLE II.

*Influence of starvation upon the creatine content of rabbit muscle.**

GROUP	ANIMAL	LENGTH OF STARVATION	CREATINE CONTENT OF MUSCLE†	CREATINE ELIMINATED IN URINE DURING STARVATION	AMOUNT OF INITIAL BODY CREATINE ELIMINATED IN URINE	CREATINE UNACCOUNTED FOR
		days	per cent	grams	per cent	per cent
A.....	41	10	0.610	1.28	38	12
	37	6	0.608	0.73	26	15
	41	7	0.564	0.22	5	4
	20	13	0.556	0.62	19	13
	38	15	0.552	0.90	28	14
Average.....		10	0.578	0.75	23	12
B.....	23	14	0.504	1.30	49	8
	40	12	0.488	2.02		
	36	22	0.462	1.55	48	5
	24	11	0.461	0.91	30	24
Average.....		15	0.479	1.48	42	12
C.....	43	17	0.456	1.51	50	15
	39	24	0.451	1.91	46	21
	42	19	0.435	1.20	35	27
	16	27	0.410	0.89	21	34
Average.....		22	0.438	1.38	38	24

* In this table, only those experiments have been included in which moisture determinations were made in the muscle.

† Figures reduced to moisture content of 76 per cent.

To make the results comparable, the figures for the concentration of creatine have been reduced to a uniform moisture content of 76 per cent, which is approximately the normal content. With one exception (Rabbit 41), all animals were allowed to come

to the point of death. A study of this table shows that in group A, in which an increase in the content of creatine was observed, the length of the fast was comparatively short, and that much less creatine was eliminated in the urine than in group B. The high content of creatine is interpreted as due to a more rapid loss of the non-creatine containing portion of the muscle than of that containing the creatine. In group B, however, an average of twice as much creatine is lost in the urine. This loss is sufficient to cause a very decided lowering of the muscle creatine, from 0.58 to 0.48 per cent. The chief factor here is obviously the marked excretion of creatine in the urine. In group C, in which the average length of life is a week greater, we find a still further depletion of the muscle creatine, 0.44 per cent, with, however, a smaller excretion of creatine in the urine. It is evident that another factor must play a part here, and an inspection of the table shows that the amount of creatine remaining unaccounted for, that is not present in the body at death or eliminated in the urine, is twice as great in this group as in groups A and B. It is possible that in animals of group C, where the length of life was the longest, the oxidative power of the body was greater and that they were able to live more economically. The possible loss of creatine by the excretion of creatinine must not be forgotten, however.

That the decreased concentration of muscle creatine is actually due to the loss of creatine in the urine may be illustrated in another way. As pointed out above, the loss of creatine during the last days of life forms a very large part of the total elimination. By adding the creatine excreted during this terminal period to the creatine still present in the body at death, one may ascertain the creatine content and concentration of the body at the stage in the fast before the excessive elimination of creatine took place. With the ratio between the percentage of creatine in the body at these two periods and the content of muscle creatine at death, the concentration of creatine at an earlier period in the fast may be ascertained.³² To illustrate this point four

³² The method of calculation may be illustrated in case of rabbit 23. The creatine content of the body at death was 1.261 grams and the weight 0.83 kgm., thus giving a creatine concentration of 0.152 per cent. Seven days previous to death the body weight was 1.15 kgms., while the creatine

experiments have been picked from the above groups B and C, in which considerable amounts of creatine were eliminated in the urine. As shown in Table III, the concentration of rabbit 39, which fasted twenty-four days, was 0.45 per cent at death, while on the twentieth day of starvation, according to this calculation, it would have been 0.63 per cent.

Not only do these results appear to demonstrate that the decrease in the muscle creatine is due to the loss of creatine in the

TABLE III.

Influence of the rapid excretion of creatine upon the creatine content of muscle.

ANIMAL	LENGTH OF STARVATION	CREATINE ELIMINATED DURING STARVATION	CREATINE ELIMINATED DURING LAST DAYS OF STARVATION	CREATINE CONTENT OF MUSCLE AT DEATH*	CALCULATED CONTENT OF MUSCLE CREATINE* AT 4-9 DAYS BEFORE DEATH
	days	grams	grams	per cent	per cent
23	14	1.30	1.10 (7 days)	0.504	0.681 on 7th day
36	22	1.55	1.07 (9 days)	0.462	0.550 on 13th day
43	17	1.51	0.89 (4 days)	0.456	0.635 on 13th day
39	24	1.91	0.95 (4 days)	0.451	0.634 on 20th day

* Figures reduced to moisture content of 76 per cent.

urine, but they show that a very decided increase in the concentration may actually exist up to a relatively short time before death.

In their eight experiments upon starving rabbits, Mendel and Rose found that in seven instances there was a decided increase in the concentration of the creatine in the muscle. For the exception they suggest the age of the animal as a possible explanation

excreted during the last seven days of life amounted to 1.098 grams. This creatine when added to that present in the body at death gave a total of 2.359 grams, and a creatine content for the body of 0.205 per cent. The creatine content of the muscle at death, 0.504 per cent times 0.205 divided by 0.152 gave 0.681 per cent as the muscle creatine content seven days previous to death.

in view of Mellanby's³³ observation that young animals have much less creatine than adults. As the animal in question weighed 2.54 kgms., this would hardly appear to be a very satisfactory explanation. The uniformly high figures of Mendel and Rose may easily be explained by the results given in Table III. These authors have pointed out the great importance of ascertaining the moisture content of the muscle in starvation before drawing conclusions as to the content of creatine. That this is essential may be seen by inspecting Table I. In our earlier experiments we were primarily concerned with the determination of the total content of creatine in the body rather than that of the muscle; and on this account, we did not adequately appreciate this point, and hence in the earlier experiments moisture determinations were not always made. The moisture may make a difference as great as 20 per cent, and it is true that the results of Dorner³⁴ might have been explained on this basis. This would hardly suffice, however, to explain the results of Howe and Hawk.³⁵

The creatine of the urine during starvation.

Since the discovery by Benedict of the elimination of creatine in the urine during starvation, it has generally been assumed that the creatine appearing in the urine in this condition, and also in pathological conditions associated with malnutrition and loss in weight had its origin in the muscle. So far as we are aware, this has never been conclusively demonstrated. The data which have been presented on the influence of creatine in the urine upon the content of creatine in the muscle point very clearly to the generally assumed origin. The fact that the creatine eliminated in the urine during the period of starvation accounts for the reduction in the creatine content of the body as shown in Tables I, III, and IV very greatly strengthen this view. About 12 per cent still remains unaccounted for in the fast of ordinary length, but this is what we might expect from our knowledge of

³³ *Loc. cit.*

³⁴ *Loc. cit.*

³⁵ *Loc. cit.*, see also paper by Biddle and Howe: *Biochem. Bull.*, ii, p. 386, 1913, which appeared after the present paper had been submitted for publication.

the fate of administered creatine. It is possible that this is due to the excess of creatine destruction over the creatine formation.

As shown in Table V and briefly summarized in Table VI below, the urinary excretion of creatine rises very rapidly in the last days of life. Animals which live for only a short period excrete relatively larger amounts the first week than those which

TABLE IV.

Influence of starvation upon the creatine content of the body.

ANIMAL	LENGTH OF STARVATION	INITIAL CONTENT OF CREATINE IN BODY, CALCULATED, INITIAL WT. $\times 0.182$ *	RELATION TO INITIAL CREATINE			IN PER CENT OF INITIAL CREATINE		
			Creatine of body at death	Creatine eliminated in urine	Creatine unaccounted for	Creatine of body	Creatine of urine	Creatine unaccounted for
	days	grams	grams	grams	grams	per cent	per cent	per cent
37	6	2.82	1.71	0.73	0.38	59	26	15
41	7	4.26	3.87	0.22	0.17	91	5	4
17	8	3.48	2.61	0.79	0.08	75	23	2
13	9	3.59	2.60	0.58	0.41	73	16	11
44	10	3.37	1.69	1.28	0.44	50	38	12
24	11	2.99	1.27	0.91	0.81	46	30	24
40	12	3.35		2.07			62	
20	13	3.22	2.19	0.62	0.41	68	19	13
21	14	2.97	1.17	1.26	0.54	40	42	18
23	14	2.66	1.26	1.30	0.10	53	49	8
38	15	3.24	1.88	0.90	0.46	58	28	14
19	15	3.29	1.70	1.24	0.35	51	38	11
43	17	3.17	1.17	1.51	0.49	35	50	15
42	19	3.42	1.28	1.20	0.94	38	35	27
36	22	3.26	1.51	1.55	0.20	47	48	5
39	24	4.13	1.34	1.91	0.88	33	46	21
14	25	3.08	1.91	0.49	0.68	62	16	22
16	27	4.26	1.91	0.89	1.44	55	21	34

* Calculated from average data in Table VII of a previous paper, this *Journal*, xiv, p. 23, 1913.

live for a much longer period. As shown in table VI, animals living two weeks eliminated two-thirds of the total creatine excreted during the second week; those living three weeks, one-half during the last week; while those which lived close to four weeks eliminated one-third the last week. The gradual rise in the elimination of creatine was brought out very nicely in one of Benedict's earlier experiments.

TABLE V.

Rate of creatine excretion during starvation.

ANIMAL.	LENGTH OF STARVATION	TOTAL CREATINE EXCRETION	RATE OF CREATINE EXCRETION DURING STARVATION							
			First week		Second week		Third week		Fourth week	
	days	grams	gram	per cent	grams	per cent	gram	per cent	gram	per cent
37	6	0.73	0.73	100						
41	7	0.22	0.22	100						
17	8	0.79	0.79	100						
13	9	0.58	0.58	100						
44	10	1.28	0.57	44	0.71	56				
24	11	0.91	0.68	75	0.23	25				
40	12	2.07	0.59	28	1.49	72				
20	13	0.62	0.14	23	0.48	77				
21	14	1.26	0.43	34	0.83	66				
23	14	1.30	0.20	15	1.10	85				
38	15	0.90	0.26	29	0.64	71				
19	15	1.24	0.24	19	1.00	81				
43	17	1.51	0.17	11	0.69	46	0.66	43		
42	19	1.20	0.21	18	0.44	37	0.56	45		
36	22	1.55	0.23	15	0.35	23	0.97	62		
39	24	1.91	0.41	21	0.25	13	0.55	29	0.71	37
14	25	0.49	0.14	29	0.14	29	0.13	26	0.08	16
16	27	0.89	0.28	31	0.17	19	0.13	15	0.31	35

TABLE VI.

Average data on rate of creatine excretion during starvation.

NUMBER OF ANIMALS	LENGTH OF LIFE	CREATINE EXCRETED DURING STARVATION	CREATINE EXCRETED DURING LAST WEEK OF LIFE	AMOUNT OF CREATINE EXCRETION IN WEEKLY PERIODS			
				First week	Second week	Third week	Fourth week
	weeks	grams	per cent	gram	gram	gram	gram
3*	1	0.70	100	0.70			
8	2	1.20	68	0.39	0.81		
3	3	1.42	51	0.20	0.49	0.73	
3	4	1.10	34	0.27	0.19	0.27	0.37

* Does not include Rabbit 41 which was killed at the end of one week, but obviously long before it would have died.

Possible relation of creatine to creatinine.

That the elimination of creatinine falls slightly during the period of starvation has, in general, been the conclusion of the various investigators who have studied this problem. In some cases, however, the fall has been so slight as to be almost negligible. The average daily elimination for week periods in our series of eighteen experiments is given in Table VII. In some

TABLE VII.

Average daily elimination of creatinine—weekly periods.

ANIMAL	CONTROL WEEK	STARVATION			
		First week	Second week	Third week	Fourth week
	mgms.	mgms.	mgms.	mgms.	mgms.
37	55	50			
41	86	88			
17	71	59			
13	69	60	22		
44	62	82	61		
24	66	58	40		
40	63	72	60		
20	74	43	53		
21	77	63	42		
23	58	47	38		
38	49	48	81		
19	76	37	80		
43	66	66	54	58	
42	73	75	65	52	
36	65	58	54	45	
39	84	92	70	57	54
14	61	51	49	32	42
16	88	62	62	44	60

cases there appeared to be a slight rise in the elimination of creatinine during the first week of starvation, while in a number of cases there was a higher elimination during the last week of life than during the preceding week. In general, however, there was a decrease. Since the weight of the whole series of animals was quite uniform, the average data may be of interest. The average daily elimination of creatinine during the control period was 70 mgms. for the eighteen animals; for the first week of starva-

tion, 62 mgms.; for the second week, 55 mgms.; for the third, 48 mgms., and for the fourth week, 52 mgms. This fall in the excretion of creatinine was shown especially well in the experiments of Mendel and Rose.

If any relation exists between the creatine of the muscle and the creatinine of the urine, and if the suggestion made in the first paper of this series—that the constancy in the content of creatine in the normal animal affords a possible explanation for the constancy in the excretion of creatinine—is correct, we would expect that a decreased excretion of creatinine would be preceded by a lowering of the creatine storehouse. In reality such is found to be the case. Since the elimination of creatine in the urine is not excessive until the days immediately preceding death, the creatine content of the body is not markedly lowered until that time. For the series of eighteen animals, the average amount of creatine found in the body at death amounts to about 55 per cent of the original content. In other words, the total loss amounts to about 45 per cent. Since the greater part of this is lost during the last days of life, the depletion is probably not much above 15 to 20 per cent during the greater part of the starvation, and this corresponds in a general way to the decrease in the excretion of creatinine. This idea was previously suggested by one of us³⁶ as a possible explanation of the decreased excretion of creatinine in pathological conditions associated with an excretion of creatine. Furthermore, it would appear evident from the recent investigations of Chisolm³⁷ that in these same conditions there was a decrease in the creatine content of the muscle. Further analyses of human muscle appear particularly desirable, especially those associated with an excretion of creatine.

It seemed important to ascertain if the creatinine eliminated during starvation would account for the creatine not present in the body at death or previously eliminated in the urine, *i.e.*, the creatine which has been tabulated as unaccounted for. Such data are given in Table I. During a long period of starvation, the creatinine calculated as creatine is considerably in excess (about 20 per cent) of the creatine which remains unaccounted for. Although this excess may represent the rate of creatine-

³⁶ Myers: *Amer. Journ. of Med. Sci.*, cxxxix, p. 256, 1910.

³⁷ Chisolm: *Biochem. Journ.*, vi, p. 243, 1912.

creatinine formation, obviously no conclusions can be drawn in light of our present lack of knowledge of the subject. In our previous communication this point was mentioned, and it was hoped that this might throw some light on the relation of creatine to creatinine; but the only relationship we have been able to elicit is that brought out in the two paragraphs below.

In their paper Mendel and Rose³⁸ pointed out that although there was no apparent relation between the creatine nitrogen or creatinine nitrogen and the total nitrogen, nevertheless, the "total creatinine" nitrogen and the total nitrogen did appear to run parallel. Our own results point to a similar parallelism as is shown in Table I. The creatine and creatinine nitrogen forms a very uniform proportion of the total nitrogen in the whole series of experiments.

Howe and Hawk³⁹ have shown that the loss of muscle tissue calculated from the excretion of creatine only accounts for about half that calculated from the total nitrogen eliminated. Benedict,⁴⁰ in discussing this topic in his original paper on the subject, pointed out that the creatine only accounted for a part of the muscle loss. Mendel and Rose have dwelt upon this point and shown that the creatinine nitrogen may in part account for this discrepancy. In Table VIII, we have calculated the average daily loss of moist muscular tissue upon the basis of the total nitrogen, the urinary creatine, and also of the urinary creatine and creatinine (in terms of creatine). The agreement between the muscle calculated from this latter source and the total nitrogen are surprisingly close, and it would seem hard to reconcile this with any other idea than that they were both closely related products of muscular metabolism.

Repeated starvation.

The results which have been obtained from the four experiments in which rabbits were starved for two ten-day periods with a feeding period of a similar length of time intervening, simply bear out the observations of the previous series of experi-

³⁸ *Loc. cit.*

³⁹ *Loc. cit.*

⁴⁰ *Loc. cit.*

ments. It was planned to kill two of the animals at the end of a third period of starvation, and the other two after they had been brought back to original weight from a similar length of starvation, and then ascertain the creatine concentration of the muscle and of the body. As may be observed from an inspection

TABLE VIII.

Flesh catabolized as calculated from the elimination of nitrogen, creatine and creatinine.

ANIMAL	FLESH CATABOLIZED CALCULATED FROM AVERAGE DAILY ELIMINATION OF		
	Total N	Creatine and creatinine as creatine	Creatine
	$\overline{N \times 28^*}$	$\overline{\times 192^*}$	$\overline{\times 192}$
	grams	grams	grams
37	36.7	34.4	23.4
41	24.1	25.7	6.1
17	33.3	32.1	19.0
13	32.5	23.8	12.5
44	41.7	37.6	24.6
24	37.3	27.3	16.0
40	37.0	38.8	23.7
20	22.4	19.6	9.0
21	25.5	29.0	17.3
23	32.8	27.3	17.9
38	28.0	27.6	11.5
19	30.5	29.4	15.9
43	32.2	25.5	12.1
42	28.3	26.9	12.1
36	26.9	25.2	13.7
39	29.1	31.1	15.4
14	13.7	13.4	3.5
16	19.3	19.0	6.3

* Calculated on the basis of rabbit muscle containing 3.6 per cent nitrogen and 0.52 per cent creatine.

tion of Table IX, three of the animals did not bear feeding after the second period of starvation. Rabbit 52 suffered from severe diarrhea, and, though it ate well, lost weight and died at the end of a week. In rabbits 48, 50, and 52, the concentration of creatine was not markedly below normal, though in rabbit 51,

which was killed nearly ten weeks after the last fast, the creatine concentration had not been restored to the normal, and, in fact, was very much lower than in the other three instances. This fact is very interesting and points to the difficulty with which creatine is replaced, at least on a carrot diet. In this series of experiments, the correspondence between the creatine excreted in the urine and that remaining in the tissues at death is brought out very nicely.

TABLE IX.

Influence of repeated starvation on creatine content of body.

ANIMAL	TOTAL LENGTH OF STARVATION	BODY WEIGHT						TIME OF DEATH AFTER LAST STARVATION
		Body weight during control period	Weight after 1st 10 days starvation	Weight after 10 days' feeding	Weight after 2d 10 days' starvation	Weight at death	Loss in weight at end of 2d starvation	
	days	kgms.	kgms.	kgms.	kgms.	kgms.	per cent	
48	20	1.76	1.39	1.59	0.97	0.99	45	1 day
50	20	1.70	1.28	1.63	0.90	0.87	47	1 day
51	20	1.72	1.32	1.58	1.12	1.43	35	74 days, killed
52	20	1.82	1.40	1.68	1.19	1.02	35	8 days

TABLE IX—CONCLUDED.

ANIMAL	MUSCLE				CREATINE CONTENT OF BODY					
	Molature	Nitrogen	Creatine	Creatine*	Initial creatine Content Body weight $\times 0.182$	Creatine of tissues at death	Creatine eliminated in urine	Creatine of urine 1st 10 days	Creatine of urine 2d 10 days and to death	Creatine unaccounted for
	per cent	per cent	per cent	per cent	grams	grams	grams	gram	grams	gram
48	76.2	3.96	0.496	0.500	3.20	1.44	1.48	0.65	0.83	0.28
50	76.8	3.70	0.451	0.487	3.09	1.56	1.34	0.57	0.77	0.19
51	76.3		0.427	0.433	3.13	2.25	0.90	0.41	0.49	0
52	82.5	2.84	0.354	0.486	3.31	0.96	1.70	0.51	1.19†	0.65

* Figures reduced to 76 per cent molature.

† 0.79 gram of this eliminated during last 8 days of life.

DISCUSSION.

The experiments above described indicate that the creatine concentration of the muscle in the rabbit is first increased during starvation, then subsequently decreased. The increase is apparently due to the removal of the non-creatine portion, *e.g.*, glycogen, fat, etc., of the muscle more rapidly than that containing the creatine. The elimination of creatine in the urine increases with the length of the fast and in the days preceding death relatively large amounts are eliminated. This results in a depletion of the creatine supply of the body and in a decreased content of muscle creatine. That this is the case may be seen by inspecting the data given in Table III. The amount of creatine lost in the urine during the last four days of life may be sufficient to cause the creatine content of the muscle to drop from 0.63 to 0.45 per cent, though the creatine content of the muscle may be as high as 0.63 per cent on the twentieth day of fasting. It is believed that this explains the uniformly high results of Mendel and Rose. In animals fasting for a considerable period, three to four weeks (Table II), there is a loss of creatine which is not so well accounted for by the creatine eliminated in the urine. Possibly the oxidative powers of the body are a little greater, and this still further depletes the creatine of the muscle. The loss of creatine in the urine is still, however, the most important factor in causing a depletion in the creatine content of the muscle.

The fact that the creatine content of the muscle is so clearly dependent upon the amount and rate of creatine excretion in the urine, is very convincing proof of the origin of the creatine of the urine. When this is added to the fact that the creatine eliminated in the urine—often 40 to 50 per cent of the initial content—very largely accounts for the depletion in the creatine content of the body caused by starvation (Table IV), there would appear to be little doubt as to the source of the urinary creatine. Furthermore, the elimination of creatine is always the greatest during the last days of life, when the greatest destruction of muscle tissue, as evidenced by the increased excretion of nitrogen, occurs.

As shown in Table VI, the rate and amount of creatine excreted during starvation are determined to a considerable extent

by the length of life of the animal, which in turn is probably dependent upon its nutritive condition. For animals living two weeks, two-thirds of the total creatine eliminated is excreted the second week; for animals living three weeks, one-half the third week; while animals living four weeks eliminate one-third the fourth week.

In light of the above data, we believe there can be little doubt as to the origin of urinary creatine in pathological conditions associated with malnutrition and loss in weight, especially in view of the observations of Chisolm that there is a decrease in the content of muscle creatine, at least in some of these conditions. We have been hoping to verify Chisolm's observations in this regard, but as yet have been unable to secure sufficient material.

The excretion of creatine by growing animals and during lactation in women cannot entirely be reconciled with the above. That children eliminate creatine we have been able to verify. We hope to make the elimination of creatine by children and by nursing women the topic of a subsequent communication.

The evidence which has been thus far presented to show a relationship between creatine and creatinine is still unsatisfactory, but the data upon which several workers have declared the independence of these substances in metabolism are even more scanty. It has been stated that the only relationship which has been shown to exist is a chemical one.

In the previous paper of this series, it was pointed out that the creatine content of the muscle for a given species is remarkably constant, and it was suggested that this might be the underlying factor in the constant excretion of creatinine. Furthermore, it was shown that for rabbits of a fairly uniform body weight, the ratio which existed between the total creatine of the body and the average excretion of creatinine was very constant. It was further noted that animals having a high content of muscle creatine eliminated a correspondingly large amount of creatinine. A small amount of evidence bearing upon this question has been adduced in the present paper. It has been pointed out that the elimination of creatinine gradually decreases as starvation progresses—and this has been the observation of other workers—and further, that this decreased excretion of creatinine follows in

a general way the decline in the creatine content of the body. In Table I, figures representing average data for the whole period of starvation are given which show that whereas neither the creatine nitrogen nor the creatinine nitrogen forms any very uniform part of the total nitrogen of the urine, nevertheless the nitrogen from both of these sources, when taken together, makes a very uniform part of the total.

Various workers have shown that the muscle disintegration as calculated upon the creatine elimination only accounts for about half that figured upon the basis of the total nitrogen. Mendel and Rose have suggested that the creatinine nitrogen may in part account for this discrepancy. Data bearing on this point are given in Table VIII. With a few unexplainable exceptions, the average daily loss in muscle tissue, as calculated upon the basis of the total nitrogen and that of the creatine and creatinine (as creatine) are surprisingly close. From these data alone it would appear that both creatine and creatinine had a common origin, viz., in the muscle tissue.

It has been stated that since it is so difficult to completely convert creatine to creatinine or creatinine to creatine outside the body, it is illogical to believe such a conversion possible in the body. It must be admitted by the supporters of this idea, however, that it is very easy to produce a slight conversion in either direction *in vitro*; in fact, unless special precautions are taken, it is difficult to prevent a slight conversion, e.g., of creatine to creatinine. This slight conversion is quite sufficient to explain the origin of creatinine. After the administration of creatine to man, Folin⁴¹ did not detect an appreciable change in the elimination of creatinine, but van Hoogenhuyze and Verploegh,⁴² Towles and Voegtlin⁴³ and the present writers⁴⁴ have observed an appreciable increase in the excretion of creatinine. In our experiments, this averaged about 3 per cent.⁴⁵ It is well known that the crea-

⁴¹ Folin: *Hammarsten's Festschrift*, III, 1906.

⁴² van Hoogenhuyze and Verploegh: *Zeitschr. f. physiol. Chem.*, lvii, p. 131, 1903; also Pekelharing and van Hoogenhuyze: *ibid.*, lxix, p. 407, 1910.

⁴³ Towles and Voegtlin: this *Journal*, x, p. 479, 1912.

⁴⁴ Paper to appear shortly.

⁴⁵ Since the present paper was written, Prof. S. R. Benedict has informed us that he has obtained similar results in experiments on dogs, the details of which are to appear at an early date.

tine of the muscle is only in very loose combination, as evidenced by the ease with which it may be extracted with water. Shaffer and Reinoso⁴⁶ have stated that muscle does contain small amounts of creatinine, 1 to 6 mgms. per 100 grams of dog muscle, amounts which they believe sufficient to account for the amount of creatinine excreted in twenty-four hours. We have made similar observations with methods which we believe preclude any conversion of creatine to creatinine. According to our figures, about 6 mgms. creatinine are present in 100 grams of fresh rabbit muscle, about one-hundredth as much creatinine as creatine. This is sufficient to account for 35 mgms. of creatinine on the basis of a body creatine content of 3.5 grams or about half the daily elimination of creatinine. Since, as already noted, creatine is only in very loose combination in the muscle, it is not difficult to believe that it is acted upon in the same way as creatine administered subcutaneously. A conversion of between 2 and 3 per cent (2.8 per cent for five rabbits with a uniform creatine-creatinine ratio) per day would entirely account for all the urinary creatinine. We have been able to demonstrate that an enzyme capable of producing this change is present in the muscle. Further discussion of this subject will be taken up in a subsequent paper.

CONCLUSIONS AND SUMMARY.

The creatine content of rabbit muscle is relatively increased in the early part of starvation, but decreased at the close of the starvation, owing to the great loss of creatine in the urine during this period.

The creatine appearing in the urine during starvation is derived from the muscle tissue, and there appears to be little doubt that this is true in pathological conditions associated with malnutrition and loss in weight.

The question of the origin of creatinine is discussed. It is believed that the evidence to support the contention that creatine and creatinine are independent in metabolism is entirely inconclusive, while the observations lending support to the older idea of the origin of the urinary creatinine from the muscle creatine are quite numerous, though as yet not entirely complete.

⁴⁶ Shaffer and Reinoso: *Proc. Soc. of Biol. Chem.*, this *Journal*, vii, p. xxx, 1910.

THE INFLUENCE OF CARBOHYDRATE FEEDING UPON THE CREATINE CONTENT OF MUSCLE.

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(Received for publication, June 19, 1913.)

It has been shown by Cathcart¹ in experiments on men, by Mendel and Rose² in experiments on rabbits, and by one of us³ in experiments on dogs, that the elimination of creatine during inanition can be reduced to a negligible quantity by the administration of carbohydrate. On the basis of this observation, Cathcart and Taylor,⁴ Wolf and Osterberg,⁵ and Mendel and Rose⁶ have found that depriving the body tissues of carbohydrate with the aid of phlorhizin in experiments on dogs will bring about an excretion of creatine. The action of carbohydrate in this connection was previously recognized by Shaffer and Coleman,⁷ who pointed out the value of diets high in caloric value and especially rich in carbohydrate in the treatment of human typhoid fever. They showed that in this way the loss of creatine could either be entirely prevented or reduced to a minimum, and for the reason that the body protein was spared by the carbohydrate.

Cathcart⁸ believes that the appearance of creatine in the urine is more or less intimately connected with disturbances in the

¹ Cathcart: *Journ. of Physiol.*, xxxix, p. 311, 1909.

² Mendel and Rose: this *Journal*, x, p. 213, 1911.

³ A study of the influence of carbohydrate (also of fat and protein) upon the excretion of creatine by starving dogs was suggested to one of us by Professor Mendel in 1907. Several experiments were carried out in the Sheffield Laboratory, though the details have never been published (see Myers: *Amer. Journ. of Med. Sci.*, cxxxix, p. 256, 1910).

⁴ Cathcart and Taylor: *Journ. of Physiol.*, xli, p. 276, 1910.

⁵ Wolf and Osterberg: *Amer. Journ. of Physiol.*, xxviii, p. 71, 1911.

⁶ *Loc. cit.*

⁷ Shaffer and Coleman: *Arch. of Int. Med.*, iv, p. 538, 1909.

⁸ *Loc. cit.*

metabolism of carbohydrate, while Mendel and Rose⁹ state that without question the metabolism of creatine is intimately associated with carbohydrate metabolism, but conclude that it is difficult to form any chemical picture of the influence carbohydrate may have in preventing the excretion of creatine.

It occurred to the writers that a study of the creatine content of the muscle in animals fed upon carbohydrate for a considerable period might throw light on this interesting problem. The methods employed were the same as those described in the preceding paper.¹⁰

EXPERIMENTAL PART.¹¹

Five experiments are reported on animals fed almost exclusively on carbohydrate for from four to fifty-three days, and one experiment on an animal living twenty-nine days, to which creatine was given in addition to the carbohydrate. The carbohydrates employed were principally soluble starch and cane sugar. They were administered in solution with the aid of a urinary catheter employed as a stomach tube. In some cases arrowroot starch was employed to a small extent, while in an endeavor to prevent diarrhea, agar agar, protein-free milk, filter-paper and bone ash were used to a limited extent during the latter part of the feeding. These measures, however, were not efficacious in preventing diarrhea. Rabbit 46, which lived for fifty-three days, was free from diarrhea until the last three days of life. Occasionally, small amounts of carrots were given, though the amount of nitrogen administered in this way was negligible.

A summary of the results of these experiments may be found in Table I, although the results of one experiment, rabbit 46, have been tabulated in greater detail in Table III. Experiment 57 will be discussed more fully in a subsequent paper dealing with the fate of administered creatine.

⁹ *Loc. cit.*

¹⁰ Myers and Fine: this *Journal*, xv, p. 283, 1913.

¹¹ A preliminary report of these experiments was presented to the Society for Experimental Biology and Medicine, May 21, 1913; cf. Fine and Myers: *Proceedings*, x, p. 168, 1913. We were assisted in these experiments by Mr. Adolph Bernhard.

From an inspection of Table I it will be seen that the loss in weight was much less in comparison with the length of life than in the starving animals. It is further worthy of note that the muscle had a much more normal appearance than in the case of starving animals, although in this case the extracts for the creatine estimation were practically colorless, in contrast to the light

TABLE I.

Influence of carbohydrate feeding upon the creatine content of the body.

ANIMAL	LENGTH OF CARBOHYDRATE FEEDING	BODY WEIGHT			COMPOSITION OF MUSCLE				CREATINE OF BODY AT DEATH	
		Initial Weight	Weight at death	Loss in weight	Molasture	Nitrogen	Creatine	Creatine*		
	days	kgms.	kgms.	per cent.	per cent.	per cent.	per cent.	per cent.	grams	per cent.
46	53	1.65	1.16	30	77.6	3.11	0.339	0.364	1.62	0.140
53	24	2.44	1.59	35	77.8	3.49	0.367	0.397	2.51	0.158
54	19	2.12	1.60	25	78.4	3.16	0.373	0.414	2.51	0.157
55	11	1.82	1.34	26	74.3	3.94	0.596	0.556	2.76	0.206
61	4	2.22	1.90	14	71.0	4.88	0.643	0.530	3.46	0.182
57	29	1.98	1.22	38	76.2	3.55	0.482	0.486	2.18	0.179

* Figures reduced to moisture content of 76 per cent.

TABLE I—CONCLUDED.

ANIMAL	INITIAL CONTENT OF BODY CREATINE CALCULATED IN INITIAL WT. $\times 0.182$	RELATION TO INITIAL CREATINE				IN PERCENT OF INITIAL CREATINE		
		Creatine content of body at death	Creatine excreted in urine	Creatine unaccounted for	CREATININE ELIMINATED DURING CARBOHYDRATE FEEDING TERMS CREATINE	Creatine of body at death	Creatine excreted in urine	Creatine unaccounted for
	grams	grams	grams	grams	grams	per cent.	per cent.	per cent.
46	2.97	1.62	0.44	0.91	3.11	55	15	30
53	4.44	2.51	0.82	1.11	2.34	57	18	25
54	3.86	2.51	0.23	1.12	1.21	65	6	29
55	3.31	2.76	0.10	0.45	0.67	83	3	14
61	4.04	3.46	0.06	0.52	0.29	86	1	13
57*	3.60	2.18	0.20	1.13	2.20	61	8	31

* 1.05 grams creatine given subcutaneously in equal doses over a period of 12 days; 60 per cent recovered in urine.

yellow color observed in normal and starving animals. An appreciable amount of creatine was eliminated in the urine, though in some of the experiments the greater part of this was eliminated during the last days of life.

In general, the influence of the carbohydrate feeding upon the creatine content of the muscle was very similar to that observed in the starving animals. In animals living for only a short period there was an actual increase in the creatine content of the muscle, but with the increase in the length of the period there was a decreased concentration. When the carbohydrate feeding extended to three weeks or over, the creatine concentration was lower than that observed during starvation, although the absolute

TABLE II.

Creatine content of muscle as influenced by starvation and carbohydrate feeding.

AVERAGE DATA FROM RABBITS	LENGTH OF STAR- VATION OR CARBOHYDRATE FEEDING	LOSS IN BODY WEIGHT	CREATINE CONTENT OF MUSCLE†	CREATINE ELIMINATED IN URINE	AMOUNT OF INITIAL BODY CREATINE ELIMINATED IN URINE	CREATINE UNACCOUNTED FOR
	days	per cent	per cent	grams	per cent	per cent
44, 37, 41, 20, 38*	10	35	0.578	0.75	23	12
23, 40, 36, 24*	15	45	0.479	1.48	42	12
43, 39, 42, 16*	22	48	0.438	1.38	38	24
46, 53, 54	32	30	0.392	0.50	13	28

* See Table II, preceding paper.

† Figures reduced to moisture content of 76 per cent.

amount of creatine present in the body at death was greater than in the starvation experiments. That such would be the case might have been inferred from the smaller percentage loss in weight.

A comparison of the results of three of the experiments, namely those on the animals living three weeks or over, with the starvation experiments is shown in Table II. The influence of the different factors previously mentioned is well brought out in this table. It would appear that the action of the carbohydrate in inhibiting the elimination of creatine, was dependent in large part upon the sparing action of the carbohydrate upon the body pro-

TABLE III.

Protocol table—carbohydrate rabbit 46.

DATE	BODY WEIGHT AT END OF PERIOD	AVERAGE DAILY DIET				CREATININE		CREATININE		IN PER CENT OF TOTAL N		
		Carrots	Starch or sol- uble starch	Sucrose	Water	Per week	Daily average	Per week	Daily average	Creatinine N	Creatinine N	Creatinine and creatine N
	grams	grams	grams	grams	grams	grams	mgms.	mgms.	mgms.	per cent	per cent	per cent
Dec. 24-31.....	1.63	350				0.359	51	0	0	2.9	0	2.9
Dec. 31-Jan. 7.....	1.45	50	10	5	30	0.335	48	61	9	4.5	0.7	5.2
Jan. 7-Jan. 14.....	1.44	20	15	10	50	0.392	55	47	7	11.4	1.3	12.7
Jan. 14-Jan. 21.....	1.39	15	5	10	50	0.386	55	68	11	9.3	1.6	10.9
Jan. 21-Jan. 28.....	1.35	5	15	10	50	0.358	51	26	4	7.9	0.5	8.4
Jan. 28-Feb. 4.....	1.30	20	0	10	60	0.354	51	64	6	6.5	0.7	7.2
Feb. 4-Feb. 11.....	1.23	0	5	15	70	0.307	44	35	5	20.5	2.0	22.5
Feb. 11-Feb. 18.....	1.19	0	10	15	70	0.343	49	53	8	14.0	2.0	16.0
Feb. 18-Feb. 22.....	1.20	0	10	20	90	0.159	39	81*	20	8.5	3.8	12.3
	1.16											

* Urine during last few hours of life (10 cc.) contained 25 mgms. of creatine but no creatinine.

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tein, *i.e.*, the muscle tissue, in consequence of which the muscle disintegration was much slower than in starvation.

From the creatine content of the muscle of rabbit 57, to which creatine was given subcutaneously, it would appear that the creatine of the muscle had been partially protected or possibly replaced by the administered creatine. This is further borne out by the relatively large percentage of creatine found in the body at death.

The results of the urine analyses have been tabulated in weekly periods (Table III) in the case of rabbit 46. In this experiment the amount of carrots given during the period of carbohydrate feeding was a little greater (total of 750 grams) than in the subsequent experiments. A gram and a half of nitrogen over a period of fifty-three days, however, would be a relatively unimportant consideration. The carbohydrate intake was insufficient in this case to prevent the elimination of creatine, though its excretion was restricted to a comparatively small amount, and remained fairly constant until shortly before death. The excretion of creatinine was very constant, there being comparatively little fall until the last days of life.

CONCLUSIONS.

The influence of carbohydrate feeding upon the creatine content of rabbit muscle is similar to that observed in starvation, although after a long period of feeding there may be an even greater reduction in the creatine concentration.

The decreased elimination of creatine after feeding carbohydrate is primarily dependent upon the sparing action of carbohydrate upon the muscle protein, or, in other words, is simply one phase of the sparing action of carbohydrate on protein metabolism.

THE RELATION OF GROWTH TO THE CHEMICAL CONSTITUENTS OF THE DIET.¹

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Our earlier experiments, undertaken to determine the relative value of different purified proteins in nutrition, revealed the fact that *growth* depends on nutritive conditions which are distinct from those required for maintenance.² This fact has since been confirmed by the experimental work of others, notably McCollum,³ and Hopkins.⁴ Some of the viewpoints in respect thereto have been discussed by us elsewhere.² In addition to our earlier experience with rats, the dissimilarity in the nutritive requirements of maintenance and growth have more recently been clearly manifested in experiments on mice conducted by Dr. Ruth Wheeler in our laboratories.⁵

¹ The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

² Osborne and Mendel: Feeding Experiments with Isolated Food-Substances, Carnegie Institution of Washington, Publication 156, Parts I and II, 1911; The Rôle of Different Proteins in Nutrition and Growth, *Science*, xxxiv, pp. 722-732, 1911; Beobachtungen über Wachstum bei Fütterungsversuchen mit isolierten Nahrungssubstanzen, *Zeitschr. f. physiol. Chem.*, lxxx, pp. 307-370, 1912; The Rôle of Gliadin in Nutrition, this *Journal*, xii, pp. 473-510, 1912; Maintenance Experiments with Isolated Proteins, *Ibid.*, xiii, pp. 233-276.

³ McCollum, E. V.: The Nature of the Repair Processes in Protein Metabolism, *Amer. Journ. of Physiol.*, xxix, pp. 215-237, 1911.

⁴ Hopkins, F. G.: Feeding Experiments Illustrating the Importance of Accessory Factors in Normal Dietaries, *Journ. of Physiol.*, xlv, pp. 425-459, 1912.

⁵ Wheeler, Ruth: *Journ. of Exp. Zool.*, 1913 (in press).

These well verified facts serve to raise the question as to what is the factor in the diet which is peculiarly essential for growth. Our own experiments, as well as those of the other investigators mentioned, make it clear that something further than a sufficient supply of energy-yielding food material is required to promote a normal growth. The animal cells need for their activities not only energy, but also suitable constructive material to replace the wear-and-tear therein. Furthermore the cells are concerned in the elaboration of a great diversity of complex and little understood substances such as enzymes, products of internal secretion, etc., which unquestionably play an indispensable rôle in life and may require either special antecedent products for their construction, chemical activators of some sort, or minute quantities of readily overlooked rarer elements and compounds. It is easy, yet futile at the present time, to develop detailed hypotheses respecting the almost innumerable possibilities involved. The greatest promise of success in discovering the food factors which determine successful growth lies in seeking them in some chemical constituents of such diets as have proved adequate to promote growth.

In attempting to ascertain what constitutes an adequate diet, by feeding experiments with isolated substances, we have found that our purely artificial foods—mixtures of isolated proteins, fats, carbohydrates and inorganic salts—sooner or later fail to maintain mature animals. In view of this such dietaries may naturally be expected to fail to maintain the less resistant young during their adolescent period. It is true that in several instances we have succeeded in keeping grown rats in health and in apparent nutritive equilibrium on purely artificial food mixtures over periods far longer than the experience of our predecessors had led us to expect. But the outcome has never been satisfactory in the sense of extending over what may be considered as the larger portion of the life span of an adult animal. Successful maintenance has been secured only when the animals were fed, in part at least, with foods containing our "protein-free milk," the preparation and composition of which has been detailed elsewhere.⁶ The superiority of the latter foods, compared with any

⁶ Osborne and Mendel: *Feeding Experiments with Isolated Food-Substances*, Carnegie Institution of Washington, Publication 156, 1911, Part II, p. 80.

purely artificial food mixture in repairing the depleted body weight of animals that have begun to decline on the artificial salt mixtures tested is beyond question. Instances of immediate recovery following the replacement of the inorganic constituents of the dietaries and part of the carbohydrates by the "protein-free milk" have been published, and might be duplicated in great numbers from our protocols.⁷ Even greater success is manifested in maintenance experiments in which the "protein-free milk" alone furnished the inorganic constituents of the dietary during long periods of time.⁸ The superiority of the "protein-free milk" foods over the diets containing artificial salt mixtures in the maintenance experiments also is unquestioned. Wherein the difference lies is not yet apparent. We have already pointed out that the efficiency of this adjuvant to the energy-yielding nutrients is not attributable to the minute trace of milk protein present. The fact of the greater efficiency of the natural milk product suggests that some constituent present in milk is essential for prolonged maintenance.

In our numerous experiments milk has proved to be an adequate food, both for growth and maintenance. Young rats fed solely upon the milk food which we have been accustomed to use⁹ not only have grown from infancy to full maturity, but have also given birth to litters of normal young which in turn have thriven on diets precisely like that furnished to their parents, as illustrated by chart I in the appendix. One must conclude from these facts that the milk food contains all that is essential for both growth and maintenance.

We have imitated the gross composition of this highly successful milk food by preparing mixtures of purified protein, lard,

⁷ Osborne and Mendel: Feeding Experiments with Isolated Food-Substances, Carnegie Institution of Washington, Publication 156, Part II, 1911; The Role of Different Proteins in Nutrition and Growth, *Science*, xxxiv, pp. 722-732, 1911; The Role of Gliadin in Nutrition, this *Journal*, xii, pp. 473-510, 1912.

⁸ Osborne and Mendel: Maintenance Experiments with Isolated Proteins, this *Journal*, xiii, pp. 233-276, 1912. Successful feeding trials extending over six months with similar diets on mice have been conducted by Dr. Wheeler; *Journ. of Exp. Zool.*, 1913 (in press).

⁹ The food is in the form of a paste consisting of milk powder, 60 per cent; starch, 12 per cent; lard, 28 per cent (see p. 318).

starch and "protein-free milk." Such foods have been singularly efficient in promoting growth of young rats. Individual animals vary in respect to their capacity to grow on this food, a few stopping after sixty days of growth, others continuing to grow for one hundred days or more. After normal growth stops, the animals may remain at constant weight for a few days, or grow very slowly, and then suddenly decline and die unless a change is made in the diet. Both interesting features of these experiments, namely, the excellent earlier growth and the ultimate failure, are exemplified in charts II and III, where they may be compared with the almost invariable complete success that attends the use of the milk food. The conclusion seems inevitable, therefore, that the "protein-free milk foods" are deficient in, or completely lack, something which milk contains and which is indispensable for perfect growth.

This ultimate inhibition of growth, and nutritive decline in our feeding trials with the mixtures of isolated food stuffs is clearly connected with the diet factors. Our milk food has invariably brought prompt recovery and continuation of normal growth (see charts II and III). Even brief periods of milk feeding suffice to replenish, or provide, or permit to develop, that non-protein factor in the lack of which, cessation of growth ensues. If rats are allowed to grow on our "protein-free milk" food for some time, but are given milk food for a short period before growth ceases, or even after the decline has begun, the return to the "protein-free milk" food may again be attended with a long period of successful growth until there sets in a second inhibition, or decline, which can likewise be averted, or repaired by further exhibition of milk food.

All the essential factors for growth must be present in the diet if normal growth is to occur and continue. Failure to grow may result from a variety of factors some of which, like a deficiency of protein, or carbohydrate, or inorganic salts, or an inappropriate type or mixture of these nutrients, are apparent.¹⁰ What light does the experience thus far accumulated throw upon the nature of the essential substance, if there be such? Is it organic or inorganic, or both?

¹⁰ See further our discussion of the subject in *Zeitschr. f. physiol. Chem.*, lxxx, pp. 307-370, 1912.

We have already noted that ultimately failure invariably ensues when rats furnished a diet containing an adequate protein and "protein-free milk" have made a considerable part of their natural growth at a normal rate. Since no such failure to grow is observed when young rats are fed with the milk food, and since also those that have ceased to grow on the "protein-free milk" diet or have declined are promptly restored to satisfactory conditions of growth by the use of milk, it is evident that the latter contains something which our "protein-free milk foods" lack. It seems probable that the missing substance is organic in nature; for the "protein-free milk" may be presumed to contain all of the inorganic constituents of the milk. Nevertheless, in view of the limitations of our knowledge regarding minute quantities of elements which may play an important part in nutrition, hasty generalizations in this direction are scarcely permissible.

In a recent paper¹¹ we gave charts showing very considerable growth, at a normal rate, of the young white rat when supplied with a diet consisting solely of purified starch, lard, protein, lactose and inorganic salts; or, in other words, with foods containing our so-called "artificial protein-free milk," the preparation of which is described in the communication referred to. We also reported normal growth for a relatively long time on a similar diet in which the fat was replaced by carbohydrate. The number of these experiments, while not large, was sufficient to show that growth can be made on such diets; and the fact that they were conducted at different times and with several batches of food made with chemicals of different origin, excluded the possibility of error due to any accidental incorporation in the food of substances other than those they were intended to contain. These results were so different from any we had previously obtained with purely artificial diets¹² that we at once proceeded to confirm them by new experiments conducted on a much larger scale.

The chemicals used in making the first lot of "artificial protein-free milk" (designated I) were ordinary laboratory preparations

¹¹ Osborne and Mendel: Beobachtungen über Wachstum bei Fütterungsversuchen mit isolierten Nahrungssubstanzen, *Zeitschr. f. physiol. Chem.*, lxxx, pp. 307-370, 1912.

¹² Osborne and Mendel: Growth and Maintenance on Purely Artificial Diets, *Proc. Soc. for Exp. Biol. and Med.*, ix, p. 72, 1912; Beobachtungen über Wachstum bei Fütterungsversuchen mit isolierten Nahrungssubstanzen, *Zeitschr. f. physiol. Chem.*, lxxx, p. 356, 1912.

of good quality. Later we used Kahlbaum's preparations, and with the foods containing the "artificial protein-free milk" thus made (which we designate II) in many cases we obtained growth quite comparable with that previously secured with foods of similar character, but containing the natural "protein-free milk." The outcome of all our growth experiments with the "artificial protein-free milk" food mixtures I and II are shown in chart IV. For the new experiments special care was taken to use chemicals of a high degree of purity, as ascertained by careful analyses. To our surprise the "artificial protein-free milk," III, made with these purer chemicals failed in every case but one to promote more than slight growth (see chart V). Since the only apparent difference in the conditions under which these later trials were conducted was the greater purity of the chemicals used, our attention was at once turned to those inorganic elements which have been found in animal tissues in traces, but of the need of which in the diet nothing has as yet been learned. Traces of such elements may have been present as impurities in the chemicals first employed.

We accordingly made another preparation of "artificial protein-free milk," IV, to which traces of iodine, manganese, fluorine, and aluminium were added. The composition of these different preparations of "artificial protein-free milk" is shown by the table on following page.

Without a guide as to the proper amount of these "traces" of inorganic elements to add, the quantities in IV were chosen arbitrarily. These might, therefore, be either too much, or too little, in respect to any one or all of the additions.

An inspection of chart V shows plainly that much better growth was secured with preparation IV than with III. The findings may be summarized by saying that with "artificial protein-free milk" mixtures we have, under certain conditions, obtained a very considerable growth which, nevertheless, in most instances has ceased sooner than that induced by the natural "protein-free milk." The latter food, however, also invariably fails sooner or later to satisfy the nutritive requirement for growth.

McCollum and Davis,¹³ whose significant experiments have con-

¹³ McCollum, E. V. and Davis, M.: The Influence of the Composition and Amount of the Mineral Content of the Ration on Growth, Proc. of Amer. Soc. of Biol. Chem., this *Journal*, xiv, p. xl, 1913:

Composition of "artificial protein-free milk."

(Quantities used to make sufficient of the mixture to prepare 1 kgm. of food.)

	I, II,* III†	IV
	grams	grams
CaCO ₃	13.48	13.48
MgCO ₃	2.42	2.42
Na ₂ CO ₃	14.04	3.42‡
K ₂ CO ₃	14.13	14.13
H ₂ PO ₄	10.32	10.32
HCl.....	12.75	5.34‡
H ₂ SO ₄	0.92	0.92
Citric acid + H ₂ O.....	10.10	11.11§
FeCl ₃ ·1½H ₂ O.....	0.634	0.634
KI.....		0.0020
MnSO ₄		0.0079
NaF.....		0.0062
K ₂ Al ₂ (SO ₄) ₂		0.0024
Lactose.....	246.0	246.0

* Prepared from Kahlbaum chemicals.

† Prepared from specially analyzed chemicals.

‡ The slight differences in the amounts of these compounds used in salt mixture IV, in contrast with I, II, and III, are due to the fact that in the latter, allowance was made for the sodium chloride produced by the neutralizing process in the preparation of our "protein-free milk." This addendum was omitted in IV in order to make its composition conform still more closely to that of the milk salts as such.

§ The small variations in the amounts of citric acid added are due to the fact that they were inadvertently made to correspond with two different analyses reported in the literature.

firmed ours in showing the possibilities of very considerable growth on the "artificial" dietaries, likewise appear to have encountered this cessation of growth for they state:

Rats grow normally during seventy-five to one hundred days on a ration consisting of pure casein, 18 per cent, dextrin, agar-agar and salt mixtures giving an inorganic content closely similar to either milk or egg yolk, and on certain other salt mixtures, in about the proportions found in milk and in eggs. With the same organic ration, fed with a salt mixture giving the ration an inorganic content closely similar to that of the wheat kernel, there is a complete suspension of growth. . . . Normal growth has been secured during seventy days on a ration of casein, 34 per cent, dextrin, agar-agar and a salt mixture giving an inorganic content similar in composition and quantity to that of dry skim milk.

The trenchant fact that failures can be averted or repaired by the use of milk foods leads to the inquiry wherein our "protein-

free milk" food differs from the conspicuously successful "milk food." The composition of three typical food mixtures is given for comparison.

	C		E		M
	per cent		per cent		per cent
Casein.....	18	Edestin.....	18	Milk powder*....	60
Starch.....	29	Starch.....	26	Starch.....	12
Lard.....	25	Lard.....	28	Lard.....	28
Protein-free milk	28	Protein-free milk	28		

*This product is the "Whole Milk Powder" supplied by the Merrell-Soule Company of Syracuse, N. Y. For the analysis see *Report of the Connecticut Agricultural Experiment Station, Food and Drug Products, 1909, p. 238.*

These contain, in every 100 grams:

	C	E	M
	grams	grams	grams
Protein.....	18.0	18.0	15.4
Lactose.....	23.8	23.8	22.3
Starch.....	29.0	26.0	12.0
Milk salts.....	4.2	4.2	3.6
Total fats.....	25.0	28.0	44.4
Lard.....	25.0	28.0	28.0
Butter.....	0.0	0.0	16.4
Moisture.....	0.0	0.0	2.3

To what can we attribute the difference in the relative efficiency of the foods in promoting growth? First, not to the proteins, although these are unlike; for such evidence as we have already secured makes it extremely improbable that they are responsible for the nutritive differences. Second, not to the carbohydrate and inorganic constituents; for these are essentially alike in all the food mixtures. Third, not to the effect of the heat applied in the production of the "protein-free milk" component of the foods; for the milk powder used by us has also been subjected to an equally high temperature. An inspection of the tables discloses the fact that the foods C and E lack all those components of milk which are separated in the process of centrifugation of milk, i.e., the cream and likewise any cellular elements

(mammary gland cells, leucocytes, bacteria, etc.) removed mechanically by the centrifugal process preliminary to the manufacture of our protein-free milk, or removed by subsequent filtration processes.

In seeking for the "essential" accessory factor we have, therefore, been led first of all to supply the cream component, in the form of butter, to rats which have ceased to grow on the "protein-free milk" foods. Numerous experiments still in progress have resulted in restoring rats, which have declined on the "protein-free milk" dietaries, to a weight normal for their age, quite as rapidly as does the efficient milk food. Examples of such recoveries are presented in charts VI and VII. Chart VI shows the effect of replacing part of the lard in our "natural protein-free milk" foods with a corresponding quantity of unsalted butter. It will be observed that after the cessation of growth, or after decline in body weight, recovery and renewed growth take place with the same rapidity as when the animals receive the milk food (compare charts II and III). Chart VII furnishes similar examples of recovery of animals which had ceased to grow on diets containing "artificial protein-free milk" IV, when part of the lard of this diet likewise was replaced with butter. These results are the more striking in view of the less rapid and continued growth manifested by most of the animals fed with the "artificial protein-free milk" foods. The illustrations presented in these charts are representative of a large number of similar experiments which we have conducted. It would seem, therefore, as if a substance exerting a marked influence upon growth were present in butter, and that this is largely, if not wholly, removed in the preparation of our natural "protein-free milk." Whether or not the latter is wholly deficient in this substance cannot be determined as yet from any data which we possess. It is true that young rats are able to make very considerable growth when fed on the natural "protein-free milk" diet; but possibly this is accomplished at the expense of some reserve substance stored in the cells of the young animal. It is too early to draw inferences as to the effective substance supplied by the butter. The detailed study of these important questions is being continued by us.

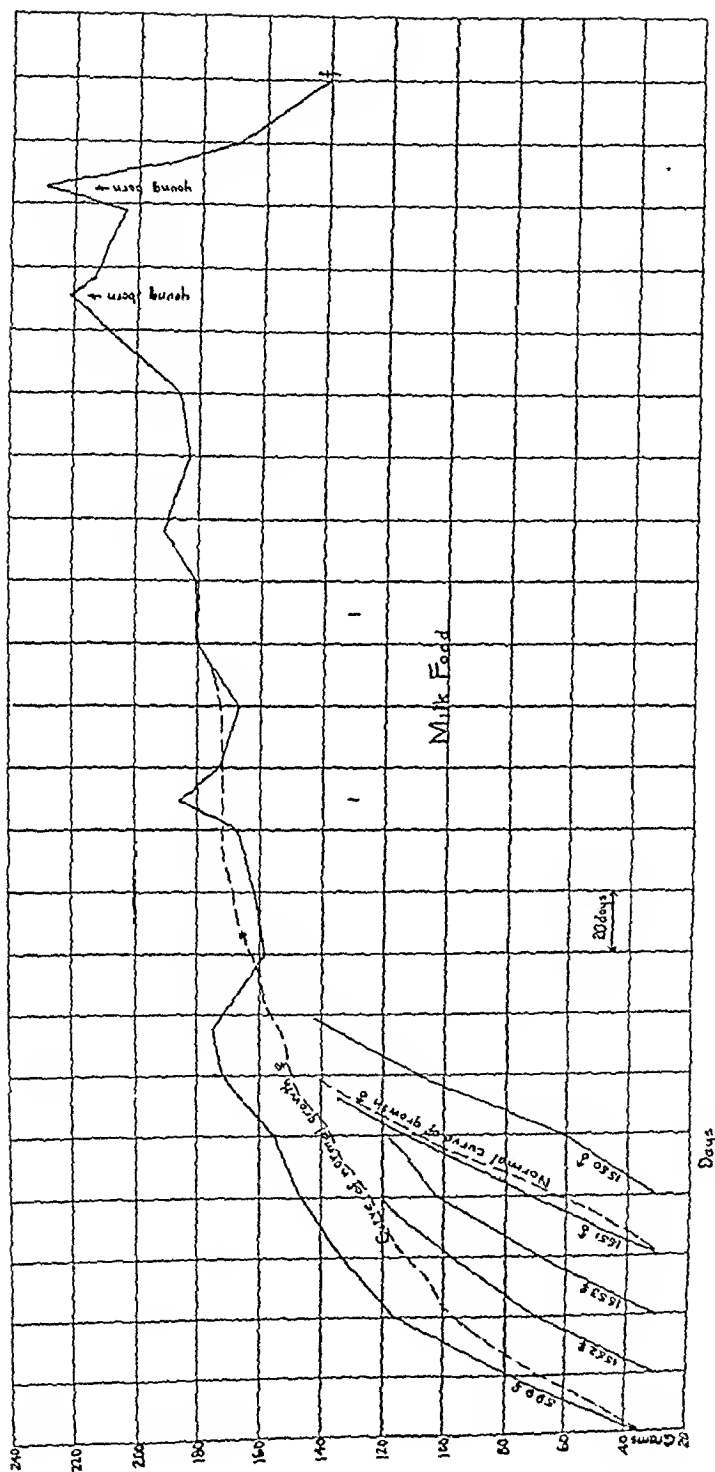


CHART I. Typical curves showing normal growth of white rats on our milk food. Rat 599 ♀, after 371 days of growth and maintenance, gave birth to two litters of young. Rats 1550, 1551, 1552 and 1553 are the young of a mother fed on the milk food from the age of 59 days for 120 days prior to their birth. The normal growth of her young, which in turn were fed from the time of weaning on the milk food, is here represented.

The ordinates represent grams of body weight, as indicated. The divisions of the abscissa represent 20-day periods.

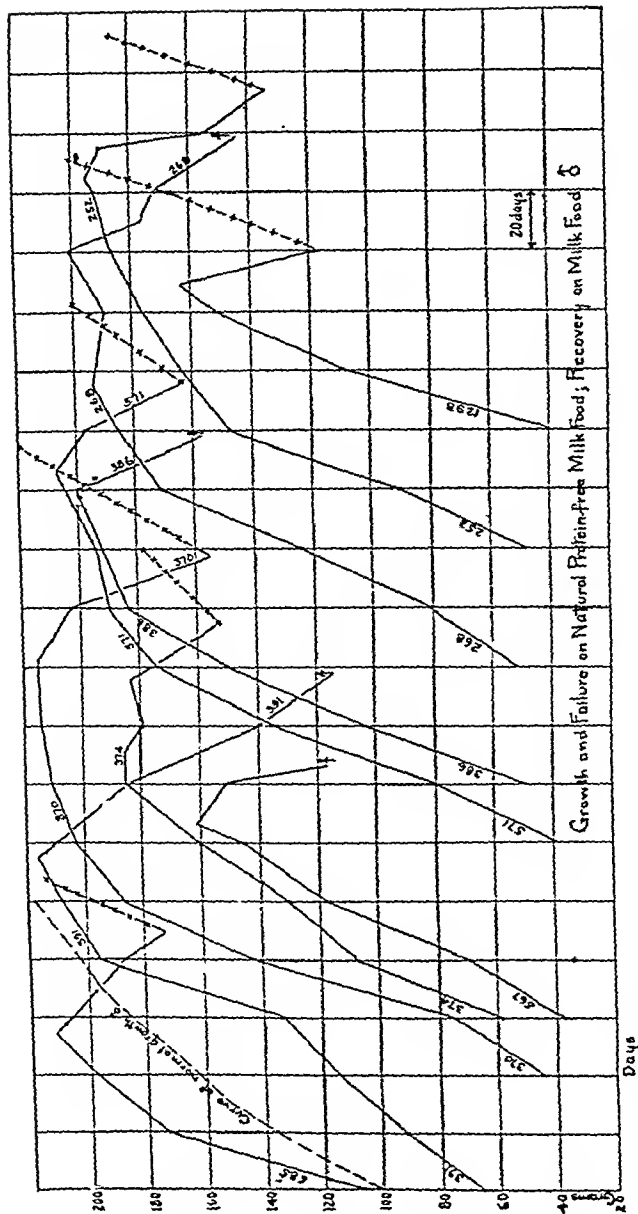


CHART 11. Curves showing normal growth of male rats during many days, followed by cessation of growth, and decline on our "natural protein-free milk" foods. In every case where our milk food replaced the earlier mixture prompt recovery followed as indicated by the interrupted lines (x-x-x-x). The "protein-free milk" foods fed to the different rats contained various proteins as follows: casein, Rats 252, 268, 370, 380, 391; glutenin, Rat 374; lactalbumin, Rat 685; maize glutelin, Rat 587; ovovitellin, Rats 571, 1298.

The ordinates represent grams of body weight, as indicated. The divisions of the abscissa represent 20-day periods.

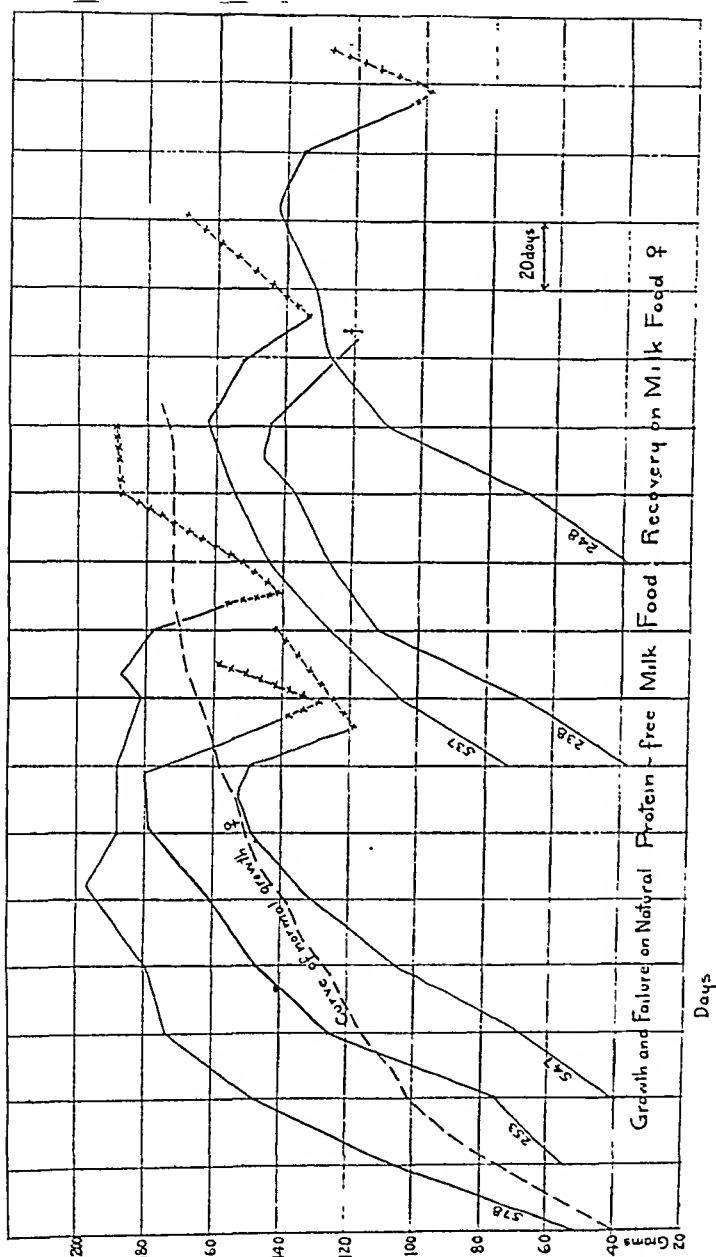


CHART III. Curves showing normal growth of female rats during many days, followed by cessation of growth, and decline on our "natural protein-free milk" foods. In every case where our milk food replaced the earlier mixture, prompt recovery followed as indicated by the interrupted lines (x-x-x-x). The "protein-free milk" foods fed to the different rats contained various proteins as follows: casein, Rat 238; edestin, Rats 248, 253; maize glutelin, Rat 547; ovovitellin, Rat 578; squash-seed globulin, Rat 537.

The ordinates represent grams of body weight, as indicated. The divisions of the abscissa represent 20-day periods.

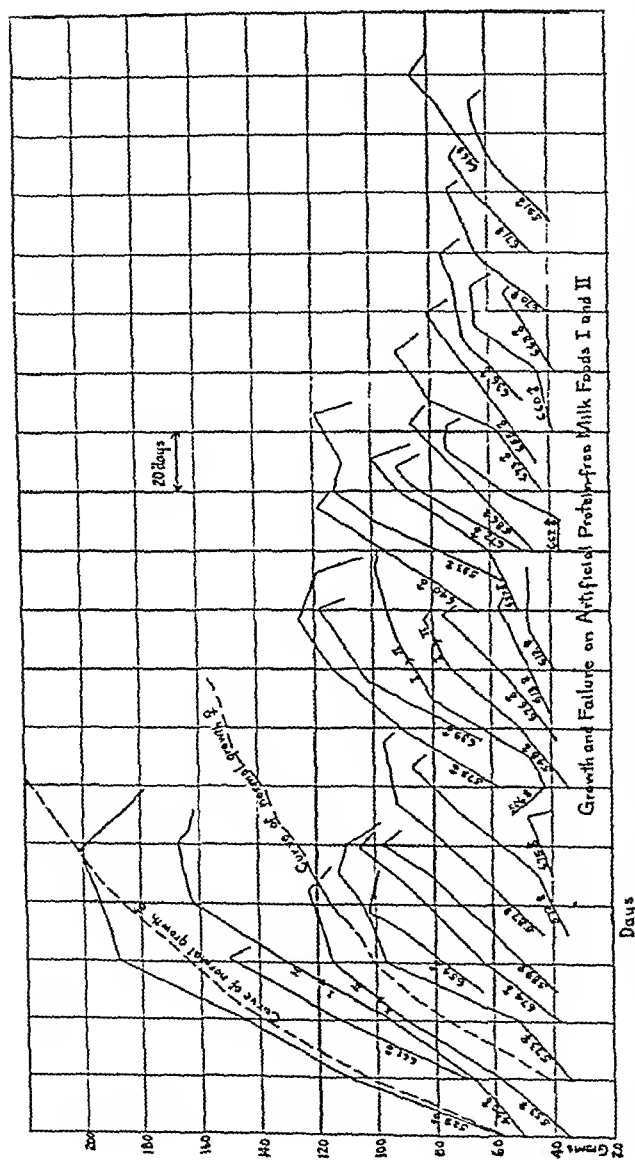


CHART IV. Curves of growth of all our rats fed on foods supplying the inorganic constituents in the form of "artificial protein-free milk" I and II. See page 317. Except where otherwise indicated in the chart, mixture II was used. It will be noted that in some cases growth was made at a rate comparable with that promoted by the "natural protein-free milk" as shown in charts II and III. In many cases the rats have more than doubled their weight. In general growth ceased sooner than when "natural protein-free milk" is fed. The ordinates represent grams of body weight, as indicated. The divisions of the abscissa represent 20-day periods.

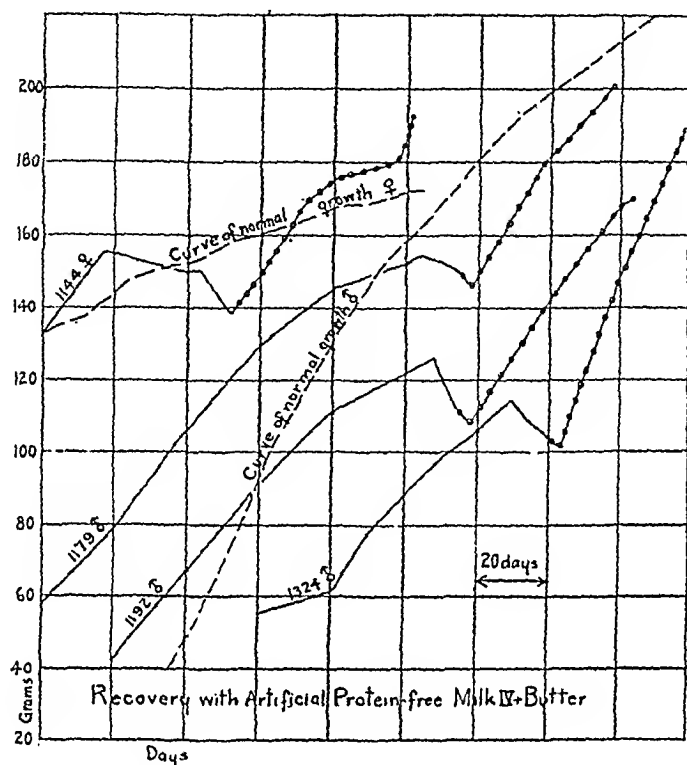


CHART VII. Curves of the body weight of rats which have ceased to grow or have declined on foods containing the "artificial protein-free milk" IV, and have recovered when part of the lard of the diet was replaced by a corresponding quantity of unsalted butter as indicated by the interrupted lines (o-o-o-o-o-o-o). The proteins furnished in the different experiments were as follows: casein, Rats 1144, 1192, 1324; edestin, Rat 1179; lactalbumin, Rat 1144.

The ordinates represent grams of body weight, as indicated. The divisions of the abscissa represent 20-day periods.

STUDIES ON THE METABOLISM OF AMMONIUM SALTS.

I. THE ELIMINATION OF INGESTED AMMONIUM SALTS IN THE DOG UPON AN ADEQUATE MIXED DIET.

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(Received for publication, June 23, 1913.)

The early recognition of the metabolic significance of ammonium salts has resulted in an extensive literature upon the behavior of these salts introduced into the organism. Upon the results obtained by Neubauer, Lohrer, v. Knierem, Coranda, Salkowski, Feder, Munk, Schmiedeberg and Walter, Hallervorden, Pohl and Munzer, Nencki, Salaskin, etc.,¹ rests for the most part our present conceptions regarding the behavior and function of ammonium salts in the animal body. In general the prime object of these experiments was the determination of the relation of ammonium salts to urea formation and the conclusions reached are too well known to need detailed repetition. It will be sufficient to state that all ammonium salts do not lead to urea formation in equal degree, thus those ammonium compounds that are the salts of organic acids may be completely transformed to urea, whereas ammonium salts of inorganic acids are only partially converted to urea, the remainder being eliminated as ammonium salts.

Apart from the relation of urea formation the influence of ingested ammonium salts has received only scanty attention until very recently. In the literature one may find a diversity of opinion as to the extent of urea formation from ammonium chloride for example. This lack of uniformity has arisen from the fact that different investigators maintained unlike experimental conditions, such for example as diet, one employing animals receiving a full mixed diet, another using dogs in a state of inanition. In

¹ For a discussion of the problem, see Hammarsten: *Text Book of Physiological Chemistry*, 1911.

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one instance in particular should attention be drawn to the experimental conditions existing. Feder² had shown that a major portion of ammonium chloride ingested by a dog reappeared in the urine as an ammonium salt. Munk³ criticized Feder's results because fasting animals were employed. In his own investigation Munk used well-fed dogs only and in order to make the dog's urine resemble in alkalinity that of the rabbit (in which animal urea formation from inorganic ammonium salts is greater than in the dog) relatively large quantities of sodium acetate were given before and during the period of ammonium chloride administration. By this means he was able to demonstrate that as much as 53.55 per cent of the introduced ammonium salt reappeared in the urine as urea.

In view of the discordant opinions concerning the subject a systematic study has been made of the elimination of ammonium salts in dogs maintained upon a constant mixed diet. It was intended that the results obtained should serve as a basis for comparison of ammonia elimination under other experimental conditions of diet.

Methods. The observations were made upon two full-grown bitches accustomed to metabolism experiments. One of these animals was normal in every respect while from the other there had been removed,⁴ several months previous to the periods of observation, a portion of the intestine for purposes bearing no relation to the present investigation. Both were fed upon a constant mixed diet consisting of fresh meat, cracker meal and lard, with a sufficiency of nitrogen and fuel value. The water intake was also constant. The ammonium salts were fed in gelatin capsules, the nitrogen content of which was too small to require consideration even though at times as many as six capsules were given per day. The ammonium salts, all Kahlbaum preparations, were analyzed for nitrogen and were then kept in glass stoppered bottles. The urine was divided into twenty-four-hour periods by catheterization, precautions being taken to prevent cystitis. Evidence of cystitis was never observed. Total nitrogen and ammo-

² Feder: *Zeitschr. f. Biol.*, xiii, p. 256, 1877.

³ Munk: *Zeitschr. f. physiol. Chem.*, ii, p. 29, 1878-9.

⁴ This animal was Dog A of previous experiments, cf. Underhill: *Amer. Journ. of Physiol.*, xxvii, p. 366, 1911.

nia nitrogen only were estimated. The urines obtained were always markedly acid to litmus.

From the data contained in Tables 1 and 2 it is apparent that when comparable quantities of nitrogen (approximately 1 gram) are orally introduced, in the form of ammonium salts of certain organic acids, into dogs maintained under constant dietary conditions none of the nitrogen reappears in the urine in the form of ammonium salts. This constitutes a direct confirmation of

TABLE 1.

Dog 1.

Experiments with ammonium lactate and ammonium citrate.

DATE 1910	BODY WEIGHT	URINE				REMARKS
		Volume	Specific gravity	Total N	Ammonia N.	
March	kilos	cc.		grams	gram	
11	6.9	200	1.020	4.23	0.17	The daily nitrogen intake in the food during this period amounted to 4.22 grams.
12	6.9	195	1.022	4.20	0.20	
13	6.9	180	1.020	4.29	0.19	
14	6.8	180	1.026	4.36	0.24	
15	6.9	200	1.030	5.24	0.22	{ 10.41 grams ammonium lactate = 1.07 grams N were fed in 5 equal portions.
16	6.9	130	1.040	4.37	0.19	
17	6.9	180	1.020	4.27	0.19	
18	6.8	175	1.035	5.64	0.22	{ 9.07 grams of ammonium citrate = 1.10 grams N were fed in 5 equal portions.
19	6.9	220	1.035	4.25	0.20	
20	6.9	125	1.040	4.14	0.23	

some of the older investigations. That the absorption of these salts was complete is attested by the output of total nitrogen. It may be inferred that under the experimental conditions ammonium salts ingested in the form of the lactate, acetate, butyrate and valerianate are completely transformed to urea. Moreover, in spite of the fact that a fairly large portion of the small intestine had been removed from Dog 2 no evidence of any difference from the normal animal could be detected in the reaction under discussion. In neither animal were abnormal symptoms observed

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after ingestion of any of the ammonium salts with the exception of the valerianate which invariably induced nausea and vomiting. After several trials with this salt a single clear-cut result was finally obtained, Table 2, Dog 2. As may be seen from Table 3

TABLE 2.

Dog 2.

Experiments with ammonium acetate, ammonium butyrate and ammonium valerianate.

DATE 1910	BODY WEIGHT	URINE				REMARKS
		Volume	Specific Gravity	Total N	Ammonia N	
March	kilos	cc.		grams	gram	
6	8.9	200	1.020	3.96	0.25	During this period the daily intake of food N amounted to 5.29 grams.
7	9.0	140	1.035	3.90	0.27	
8	9.0	155	1.026	3.75	0.25	
9	9.0	250	1.017	3.84	0.25	
10	9.0	200	1.020	4.98	0.24	{ 6.65 grams ammonium acetate = 1.08 grams N were fed in 4 equal portions.
11	9.0	180	1.020	3.99	0.24	
12	9.0	175	1.025	3.75	0.22	
13	9.0	140	1.030	3.95	0.25	
14	8.9	195	1.026	5.16	0.23	{ 10.25 grams ammonium butyrate = 1.09 grams N were fed in 6 equal portions.
15	9.0	120	1.024	3.75	0.25	
16	8.9	150	1.030	3.72	0.25	
17	8.9	140	1.035	3.75	0.23	
18	8.9	240	1.035	4.80	0.24	{ 13.36 grams ammonium valerianate = 1.08 grams N were fed in 5 equal portions.
19	8.9	125	1.036	3.78	0.25	
20	8.9	110	1.040	3.81	0.26	

ammonium carbonate behaves in manner identical with the other ammonium salts just discussed.

When ammonium salts of the inorganic acids are considered, entirely different conclusions must be drawn—see Tables 3, 4 and 5. With ammonium chloride ingestion ammonia nitrogen vary-

ing from 41 per cent of that ingested (with Dog 2, Table 5) to 47 per cent and 52 per cent (with Dog 1, Table 3) was eliminated in the urine, the period of excretion being complete within 48 hours. With Dog 2 equal quantities of the excess of ammonium

TABLE 3.

Dog 1.

Experiments with ammonium carbonate and ammonium chloride.

DATE 1910	BODY WEIGHT	URINE				REMARKS
		Volume	Specific gravity	Total N	Ammonia N	
February	kilos	cc.		grams	gram	
21	7.0	150	1.025	3.75	0.20	Throughout this period the daily diet contained 4.16 grams N.
22	7.0	160	1.028	3.85	0.26	
23	7.0	130	1.030	3.79	0.28	
24	7.1	275	1.017	3.87	0.23	
25	7.0	150	1.032	3.85	0.21	
26	7.0	150	1.033	5.19	0.26	{ 5.52 grams ammonium carbonate containing 0.95 gram N were fed, divided into 3 portions.
27	7.0	225	1.017	3.95	0.23	
28	7.0	220	1.032	6.00	0.57	{ 3.94 grams ammonium chloride = 1.02 grams N fed in 3 portions.
March 1	7.0	220	1.030	5.01	0.38	
19	6.9	220	1.035	4.25	0.20	The daily nitrogen intake in food during this period amounted to 4.22 grams.
20	6.9	125	1.040	4.14	0.23	
21	6.9	300	1.020	5.37	0.57	
22	6.8	140	1.035	3.98	0.42	
23	6.8	100	1.040	3.76	0.23	

salt were eliminated on each of the two days of excretion. In the first experiments with Dog 1 (February 28, Table 3) 36 per cent reappeared on the first day and 11 per cent on the second. The second experiment (March 21, Table 3) demonstrated that

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36 per cent of the ammonium salt ingested reappeared in the urine during the first day and 16 per cent on the second day.

After the introduction of ammonium phosphate (Table 4, Dog 1) there appeared in the urine as ammonia nitrogen about 64 per cent of that ingested. On the first day 28 per cent was eliminated, on the second 26 per cent and on the third day 10 per cent. Of the inorganic ammonium salts the nitrogen of the sul-

TABLE 4.

Dog 1.

Experiments with ammonium phosphate and sodium chloride.

DATE 1910	BODY WEIGHT	URINE				REMARKS
		Volume	Specific gravity	Total N	Ammonia N	
April	kilos	cc.		grams	gram	
8	7.0	200	1.025	4.14	0.20	The nitrogen ingested in the food daily amounted to 4.22 grams.
9	7.0	210	1.023	4.02	0.21	
10	7.0	220	1.020	4.08	0.19	
11	7.0	175	1.030	4.00	0.19	
12	7.0	225	1.048	5.46	0.51	{ 9.6 grams ammonium phosphate = 1.11 grams N were fed in 5 equal portions.
13	6.9	125	1.035	4.14	0.49	
14	6.9	125	1.030	3.60	0.32	
15	7.0	110	1.032	3.58	0.18	
16	7.0	180	1.025	3.81	0.18	{ 4.35 grams sodium chloride fed in 3 equal portions.
17	7.0	170	1.028	3.76	0.17	
18	7.0	240	0.021	3.64	0.08	
19	6.9	80	1.038	3.46	0.17	
20	7.0	100	1.034	3.65	0.18	

phate (Table 5) seems to be more completely converted into urea than any of the others tested since only 29 per cent of the nitrogen introduced as ammonium salt was eliminated in the urine as ammonia, 18 per cent appearing on the first day and 11 per cent on the second day. With all of the inorganic ammonium salts there may be noticed a slight excess of total nitrogen excreted over the average output. In general this excess of nitrogen in

the urine is observable only on the day of ingestion although with the introduction of ammonium chloride on February 28 (Table 3) the augmented total nitrogen excretion persisted during

TABLE 5.

Dog 2.

Experiments with ammonium chloride, ammonium sulphate and sodium chloride.

DATE 1910	BODY WEIGHT	URINE				REMARKS
		Volume	Specific gravity	Total N	Ammonia N	
March	kilos	cc.		grams	gram	
19	8.9	125	1.036	3.78	0.25	The daily intake of food N amounted to 5.29 grams. 4.21 grams ammonium chloride = 1.03 grams N were fed in 4 equal portions.
20	8.9	110	1.040	3.81	0.26	
21	9.0	325	1.019	5.01	0.49	
22	8.9	110	1.040	3.81	0.46	
23	8.9	175	1.030	3.90	0.26	
April						
8	9.8	150	1.030	3.80	0.26	5.01 grams ammonium sulphate = 1.03 grams N were fed in 3 equal portions.
9	9.8	145	1.032	3.87	0.25	
10	9.8	120	1.036	3.92	0.22	
11	9.8	120	1.038	3.96	0.23	
12	9.8	200	1.042	5.13	0.44	
13	9.7	125	1.030	3.89	0.37	4.51 grams sodium chloride were fed in 3 equal portions.
14	9.7	100	1.035	3.87	0.27	
15	9.7	110	1.032	3.90	0.21	
16	9.8	110	1.032	3.81	0.23	
17	9.8	100	1.036	3.69	0.23	
18	9.8	240	1.020	3.91	0.12	
19	9.8	90	1.036	3.78	0.26	
20	9.8	100	1.035	3.81	0.24	

March 1. It is apparent that the ingestion of ammonium salts of inorganic acids usually causes an output of total nitrogen in distinct excess of the usual elimination previous to the introduction of the ammonium salt. During this period a portion of the

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ingested ammonia nitrogen is retained within the organism and the query arises whether the retention of the ammonium compound is the cause of the greater output of total nitrogen. Does the ammonium salt bring about a temporary stimulation of nitrogenous catabolism? To obtain an answer to this question whether the retention of ammonium salts is responsible for the excess of nitrogen eliminated one would turn naturally to the results obtained with the introduction of ammonium salts of the organic acids since in no instance was there retention of ammonia nitrogen. Upon inspection it is seen that the results obtained here are conflicting with respect to the subject under discussion for with the citrate and butyrate excess of total nitrogen comparable with that seen with inorganic ammonium salts may be noticed. With the acetate and valerianate no change is noticeable while with the lactate the output of total nitrogen is slightly under the normal average excretion. It is probable therefore that the more prolonged retention of inorganic ammonium salts cannot be the fundamental reason for the excess of total nitrogen eliminated. On the other hand, one may conclude that some ammonium salts, whether of organic or inorganic nature, possess the property of causing the output of a small excess of total nitrogen, which may perhaps be regarded as a slight stimulation to nitrogenous catabolism.

No adequate explanation exists for the temporary retention of the ammonium salts of inorganic acids unless indeed it is assumed that these compounds are more or less toxic and therefore must be temporarily stored until the organism can eliminate them without injury to itself. It may be accepted probably that the acid radicle is responsible for this behavior and it is possible that excess of the acid in the cells requires ammonia for its storage and subsequent excretion. To determine whether an inorganic acid ingested as a neutral salt is capable of exerting any influence upon the output of ammonia nitrogen an amount of chloride in the form of sodium chloride equivalent to that ingested as ammonium chloride was introduced under the usual conditions—see Tables 4 and 5. It will be observed from these data that no influence was exerted by sodium chloride upon the total nitrogen output nor was the ammonia nitrogen elimination increased. Instead, in both instances the ammonia nitrogen excretion was diminished to one-half the usual amount.

SUMMARY.

Ammonium salts of a number of organic acids ingested by dogs maintained upon a constant mixed diet failed to increase the ammonia nitrogen output in the urine.

Introduced under comparable conditions ammonium salts of some of the inorganic acids caused a varying degree of increase in the urinary ammonia nitrogen. The experiments afford no adequate explanation for this temporary retention of the ammonium salts.

All the *inorganic* ammonium salts tested and some of those of organic nature cause a distinct excess of total nitrogen output over the normal. These salts apparently stimulate nitrogenous catabolism.

Sodium chloride fed under the experimental conditions causes a distinct lowering of the ammonia nitrogen elimination.

STUDIES ON THE METABOLISM OF AMMONIUM SALTS.

II. A NOTE ON THE ELIMINATION OF INGESTED AMMONIUM SALTS DURING A PERIOD OF PROLONGED INANITION.

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The object of the present investigation is to determine whether a dog in a poor nutritive condition especially with respect to carbohydrate store, behaves differently than a well-fed animal when ammonium salts are ingested. In order to make the body as free as possible from glycogen phlorhizin was administered, in exact accordance with the method of Lusk, for a period of three days, beginning January 19 (see table), the urine for the last two days only being collected. Upon these days a condition of total diabetes was attained. From January 19 forward no food was given, otherwise the experimental conditions existing were in every way comparable with those of the preceding paper.

It will be noted from the figures given in the table that in spite of the probable comparative paucity of glycogen the urinary nitrogenous constituents determined failed to show any definite changed relationships. The failure of any change in this respect tends to demonstrate that during prolonged starvation when the carbohydrate store is at a minimum the organism of the dog still retains the ability to eliminate ammonia arising within the body in a normal manner, namely, as urea. Moreover, when ammonium carbonate is ingested the subsequent ammonia nitrogen excretion is in every respect similar to that of the normal well-fed animal.

The ingestion of ammonium chloride was followed by a period during which the ammonia nitrogen elimination rose far above the normal and was maintained at a high level throughout the remainder of the experiment. The reason for this is problemati-

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cal. The fact that the ammonia nitrogen rose from 0.15 gram to 0.36 gram may at least be accepted as indicating that some of the ammonium salt was temporarily retained in a manner similar to that seen with the well-fed dog. With the first ingestion of ammonium carbonate little or no excessive amount of total nitrogen output is to be observed. When ammonium chloride was introduced, however, a marked rise in total nitrogen was in evidence and the former level failed to be regained. The second ingestion of ammonium carbonate also was followed by a marked augmentation of total nitrogen excretion which did not regain the former level. It appears hardly possible that these relatively large increases in total nitrogen output could have been merely coincident with the intake of the ammonium salts without bearing some definite relation to them. It seems more reasonable to assume that the ammonium salts exert a much greater action in this direction during a period when presumably the organism must be in a condition of unusual susceptibility, than is possible under normal circumstances.

CONCLUSIONS.

From the data presented it is indicated that during a period of prolonged inanition the ingestion of ammonium carbonate by the dog fails to show any increase in the urinary ammonia-nitrogen output.

Ammonium chloride, on the other hand, causes a marked increase in ammonia nitrogen which subsequently remains at a high level. The salt is responsible also for a noticeable increase in the total nitrogen output which fails to regain the former level. At this stage a second ingestion of ammonium carbonate may also bring about a significant augmentation of the total nitrogen elimination.

The ingestion of ammonium salts during prolonged inanition.

DATE 1910	BODY WEIGHT	URINE				REMARKS
		Volume	Specific Gravity	Total N	Ammonia N	
January	kilos	cc.		grams	grams	
20	12.7	475	1.060	12.72	0.74	D : N ratio = 3.77.
21	12.5	450	1.064	8.94	0.50	D : N ratio = 3.47.
22	12.3	925	1.035	11.02	0.55	
23	11.7	600	1.035	8.64	0.64	
24	11.1	450	1.033	7.92	0.45	
25	10.9	420	1.030	5.10	0.20	
26	10.6	260	1.020	3.60	0.09	
27	10.3	110	1.030	2.85	0.18	
28	10.2	190	1.020	2.91	0.19	
29	10.0	Urine contaminated.				
30	9.8	Urine contaminated.				
31	9.5	180	1.019	3.09	0.20	
February						
1	9.2	90	1.025	2.34	0.13	
2	9.0	100	1.030	2.40	0.16	{ 5.39 grams ammonium carbonate = 0.93 gram N were fed in 3 equal portions.
3	9.0	120	1.030	3.42	0.15	
4	8.9	60	1.045	2.26	0.11	
5	8.9	100	1.035	2.31	0.15	{ 3.94 grams ammonium chloride = 1.01 gram N were fed in 3 equal portions.
6	8.8	100	1.040	4.20	0.36	
7	8.6	100	1.025	3.15	0.36	
8	8.1	90	1.035	3.45	0.53	
9	8.1	100	1.030	3.96	0.34	{ 5.53 grams ammonium carbonate = 0.95 gram N were fed in 3 equal portions.
10	7.9	130	1.035	5.32	0.36	
11	7.9	120	1.040	4.96	0.32	
12	7.7	150	1.035	6.30	0.30	
						The urine was always strongly acid to litmus.

STUDIES ON THE METABOLISM OF AMMONIUM SALTS.

III. THE UTILIZATION OF AMMONIUM SALTS WITH A NON-NITROGENOUS DIET.

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The possible rôle of ammonium salts in intermediary metabolism has been emphasized by the recent communication of Grafe and Schläpfer,¹ according to whom there is a notable retention of nitrogen when these salts are ingested by dogs maintained upon diets rich in carbohydrate and fat but containing little nitrogen. It is the opinion of these investigators that under such circumstances the retained nitrogen is combined with carbohydrate to form an amino-acid complex, hence protein synthesis occurs. Abderhalden² has confirmed the general results obtained by Grafe and Schläpfer but denies the probability of a protein synthesis with ingested ammonium salts as the source of nitrogen. Grafe and Schläpfer insist that very large quantities of carbohydrates are essential in order to obtain a satisfactory demonstration of ammonia utilization and Abderhalden has employed accordingly diets of large calorific value. On the other hand Taylor and Ringer³ have shown that "the presence of carbohydrates is not an obligatory factor in the retention of nitrogen from ammonia."

The observations of Grafe and Schläpfer lead to the inference that the character of the ammonium salts employed is a matter of indifference, ammonium salts of inorganic acids, as ammonium chloride, or of organic acids, as the citrate, showing the same

¹ Grafe and Schläpfer: *Zeitschr. f. physiol. Chem.*, lxxvii, p. 1, 1912.

² Abderhalden: *Ibid.*, lxxviii, p. 1, 1912, and lxxxii, p. 1, 1912. See also Peschek: *Biochem. Zeitschr.*, xlv, p. 244, 1912.

³ Taylor and Ringer: this *Journal*, xiv, p. 407, 1913.

type of action—varying only in degree. In view of the radically different mode of elimination of the two types of ammonium salts in dogs during starvation and also upon an adequate mixed diet one might naturally expect to find a marked difference in the character of the influence exerted upon intermediary metabolism under the experimental conditions here discussed. The determination of the urinary ammonia nitrogen output under these circumstances should contribute toward the solution of the problem. Such estimations, fundamental for the question at hand, have been made neither by Grafe and Schläpfer nor by Abderhalden.

A review of the work of Grafe and Schläpfer makes it evident that criticism may be directed at the methods of procedure adopted. In their recital of the technique employed it is stated

Vom täglichen Katheterisieren wurde abgesehen, da selbst, wenn die hintere Scheidenwand nicht gespalten wird, die Gefahr einer Cystitis, die unter allen Umständen vermeiden werden musste, bei den jungen Tieren zu gross ist. Da Periode nur mit Periode, nicht Tag mit Tag verglichen wurde, konnte ohne Beeinträchtigung der Genauigkeit der Versuche der tägliche Katheterismus unterlassen werden.⁴

For the sake of clarity a portion of the protocols of Grafe and Schläpfer are reproduced on the following page.

In view of the last statement quoted above one may query why the average of the last three days of the second fore-period should be employed as a standard representative of this period. In the absence of catheterization what is the evidence that a goodly portion of the urine properly belonging to the fifteenth experimental day was not voided on the sixteenth day? The adoption of such a procedure in metabolism investigations is always unjustifiable, and it is especially so in this instance where the point of the entire study rests upon the strictest accuracy. In the partial reproduction of "Versuch II" one may look askance at the results with even more reason, for here the nitrogen elimination of the second fore-period was extremely variable. What justification is there for selecting the average of the last two days as a standard? Again, in the experimental period (not reproduced) a whole day, the eighteenth, is excluded because a por-

⁴ Grafe and Schläpfer: *loc. cit.*, p. 4.

Versuch I. Fox, männlich (Zulage von 0,5 g N als NH_4Cl).

Ver- suchs- tag Nr.	N-Gehalt der Nah- rung g	Urinmenge ccm	N-Ge- halt des Urins g	N-Ge- halt des Kotes g	N-Bilanz pro die	N-Retention verglichen mit dem Mittelwert von Vor- und Nachperiode	Bemerkungen
9.	0,0725	360 incl. Spül- wasser	1,86	—	—	—	Vorperiode II.
10.	0,0725	225 "	0,971	0,048	-0,946	Bei einer	
11.	0,0725	125 "	0,833	0,048	-0,813	N-Einfuhr von	
12.	0,0725	210 "	0,802	0,048	-0,777	0,0725 g N pro	
13.	0,0725	225 "	0,980	0,048	-0,954	die wurden in	
14.	0,0725	?	?	0,048	?	den letzten 3	An beiden Tagen
15.	0,0725	?	?	0,048	?	Tagen im	etwas Urin verlo-
16.	0,0725	580 "	0,741	0,048	-0,716	Durchschnitt	ren gegangen.
17.	0,0725	460 "	0,811	0,048	-0,786	0,849 g N	
18.	0,0725	350 "	0,85	0,048	-0,825	pro die abgegeben	
19.	0,0725	480 "	1,23	0,036	-0,753	Bei einer tägl- lichen Zer- setzung von	Hauptperiode.
20.	0,0725	480 "	1,119	0,036	-0,7319	0,837 g N und	
21.	0,0725	380 "	0,752	0,036	-0,275	einer Einfuhr	0,5 g N in Form
22.	0,0725	355 "	1,001	0,036	-0,524	von 0,5 g täglich wurden pro die 1,121 g N aus- geschieden, d. h. 0,242 g N retiniert = 50% der Einfuhr	von NH_4Cl pro die.
23.	0,0725	450 "	0,851	0,030	-0,823	Bei 0,0725 g	Nachperiode.
24.	0,0725	320 "	0,84	0,030	-0,817	N-Einfuhr	
25.	0,0725	370 "	0,821	0,030	-0,893	verliert der	
26.	0,0725	—	—	—	—	Körper pro die 0,8763 g N	

Versuch II. Hund, Ami ♀ (Zulage von 1,0 g N als NH_4Cl).

9.	0,1016	104	2,735	0,041	—	In den letzten	Vorperiode II.
10.	0,1016	450	1,268	0,041	-1,207	Tagen N-Ver-	
11.	0,1016	550	0,963	0,041	-0,902	lust des Kör-	Etwas Durchfall.
12.	0,1010	200	0,546	0,041	-0,455	pers pro die	" "
						= 0,796 bei	(20 Tr. Tinct. opii)
						Aufnahme	" "
						von 0,1016 g	
						pro die	

tion of the urine was lost, but even making this allowance the end result is probably far from correct.

Although the adoption of the method in this case would alter very little the result one is impelled to protest against the practice of taking the average of the fore- and after-periods as a measure of comparison for the experimental period. In this way any possible influence extending beyond the experimental period is overlooked.

Turning again to the description of the technique of Grafe and Schläpfer⁵ we read

Der Stuhl wurde getrennt aufgefangen und gesammelt. Da die Tiere besonders bei den Versuchen mit Chlorammonium hin und wieder Durchfall bekamen, war eine Trennung natürlich nicht immer möglich. Es wurde dann der Stuhl durch Filtration des Urins und des Spülwassers gewonnen. Bei diesem wohl nicht zu umgehenden Verfahren konnte natürlich nicht verhindert werden, dass ein Teil des Kotstickstoffs als Urinstickstoff gerechnet wird. Die Mengen, um die es sich dabei handeln kann, sind allerdings sehr klein. Hin und wieder besonders gegen Ende der Hauptperiode erbrachen die Tiere etwas. Auch hier ist natürlich eine Trennung des Erbrochenen von Stuhl und Urin oft nicht möglich. Die festeren Bestandteile der Nahrung blieben natürlich mit dem Kot, wenn soleher nicht getrennt gesammelt werden konnte, auf dem Filter zurück, während das Ammoniumsalz mit ins Filtrat ging. Wie die ausserordentlich geringe Menge des Trockenkots (Filtrerrückstand) beweist, handelte es sich, wenn überhaupt, stets um sehr kleine Menge erbrochener Nahrung. An den Tagen, an welchen Durchfall und Erbrechen vorhanden war (vgl. die Tabellen), umschliesst die N-Bestimmung im Harn also auch einen grossen Teil des nicht resorbierten Stickstoffs; die N-Werte im Urin stellen dann also Maximalzahlen dar.

It is evident from their own description that Grafe and Schläpfer recognize that their work makes little or no pretense to accuracy. Why then in view of the many acknowledged opportunities for relatively great errors should so much emphasis be laid upon the apparent retention of small quantities of nitrogen?

If the tables dealing with the other ammonium salts, ammonium acetate and citrate, are examined less criticism is warranted although here also at times the same type of error is introduced. For example, in Versuch III, in the experimental period the nitrogen for three days is questioned, and apparently the authors

⁵ Grafe and Schläpfer: *loc. cit.*, p. 4.

consider that it is only necessary to exclude these days in order to have their results assume a condition of accuracy. In the very last period of the experiment why should the nitrogen of the feces be accepted since the figures given were obtained for the second fore-period twenty-five days previously? The conclusions derived from experiments with such possibilities for error must be regarded with a certain degree of skepticism. This, in relation to the significance of the theory advanced, was the impetus for the investigation detailed below, which in a general way is a repetition of the experiments of Grafe and Schl pfer, attempts being made to eliminate all possible sources of error.

Methods. The general plan of experimentation employed by Grafe and Schl pfer was adopted. For several days previous to the actual period of the investigation the animals were allowed to fast. This period was succeeded by one in which a non-nitrogenous diet was given and the real fore-period was begun only when the urinary nitrogen excretion had attained a more or less constant level. The feces of each period were marked off by administration of carmine, and catheterization divided the urine into daily periods. No indication of cystitis was ever observed. The diet consisted of a fine grade of cornstarch, refined lard, sucrose, bone ash and a constant quantity of water. The individual foodstuffs contained no measurable quantity of nitrogen. The animals were fed twice daily, at 8.40 a.m. and 5 p.m., during the fore- and after-periods. Throughout the experimental period the food and the ammonium salt were fed in three portions, the extra period being at noon. The food was prepared fresh for each meal and was treated as follows: The starch was made into a thick paste, the sugar, lard and bone ash added and the whole thoroughly mixed. As a rule the food was fed while warm. The ammonium salt was placed within a portion of the lard and this was first fed to the dog from a spoon, the remainder of the ration usually being greedily devoured. Toward the end of the experiments the animals at times showed a marked disinclination to eat and under these circumstances it became necessary to administer the food with a spoon. This was particularly true for Dog 2, whereas with Dog 1 such a procedure was unnecessary throughout the first experiment. Diarrhoea was never in evidence. The ammonium salts were always analyzed for nitrogen just previous to the time of administration and they were then kept in glass stoppered bottles. Chloride was estimated according to the Volhard procedure, trials having demonstrated that the results obtained in this way were identical with those derived by use of the Gr ber⁴ method. The determination of the other substances was carried through by the well-known methods and calls for no further comment. The urine was acid to litmus throughout all the periods.

⁴ Gr ber: *Zeitschr. f. Biol.*, xix, p. 569.

Experiments with ammonium chloride.

In previous experiments it has been demonstrated that ammonium chloride shows a decided difference from organic ammonium salts in its mode of elimination from the body during inanition or with an adequate mixed diet. Under these circumstances it seemed especially desirable to repeat the work of Grafe and Schläpfer with ammonium chloride to determine whether the nitrogen of this salt, in the absence of food nitrogen, would show a behavior with respect to its manner of elimination at all comparable to what had been observed under the previous experimental dietary conditions. Therefore, in addition to the determination of nitrogen balances, ammonia nitrogen excretion in the urine has been followed with the idea that this estimation might indicate better the type of processes taking place than could be derived from total nitrogen determinations only. In order to follow intermediary processes even more exactly daily chloride estimations were also made. With a similar object in view the elimination of creatinine and creatine was followed.

In Tables 1 and 2 are recorded the results obtained from feeding ammonium chloride to dogs kept upon an otherwise nitrogen-free diet but with relatively high fuel value. The contention of Grafe and Schläpfer that the nitrogen of ammonium chloride may diminish the negative nitrogen balance under the experimental conditions is in nowise corroborated by the data here presented. Comparing the average balance of the fore-period with that of the experimental period little or no difference can be found. Judged by the criterion of Grafe and Schläpfer therefore all of the nitrogen of the ammonium salt would appear to have been promptly eliminated, contrary to what they have maintained for their own experiments. A closer inspection of our ammonia figures, however, will show that not all of the ammonium salt has been so promptly excreted. There is apparently a lag in the elimination of the ingested ammonia nitrogen as is indicated by the persistence of the high ammonia nitrogen output in the after-period accompanied by an output of chlorine decidedly higher than what is to be expected from the diet, judged by a comparison with the fore-period. Moreover the average total nitrogen excretion of the after-period fails to return to the normal level and in correspondence with this the average minus nitrogen bal-

ance of the after-period was also greater than the normal. Glancing at the creatinine and creatine figures one sees that it was only at the end of the ammonium salt administration that creatine appeared in the urine. The extra ammonia nitrogen eliminated if assumed to be in combination with chlorine would account for the greater portion of the chlorine excreted. It is a little surprising that in these animals with a very small chlorine intake so little of this element should be permanently retained.

These are the facts presented by the data. What interpretation may be placed upon them? It is possible that there may be more than a single explanation but the most obvious one appears to us to be as follows: Ammonium chloride, ingested by the dog, is temporarily retained whatever the state of nutrition, *i.e.*, inanition, maintenance upon an adequate mixed diet or upon a non-nitrogenous ration. If the salt is regarded as possessing a certain degree of toxicity its retention within the body for a short period is not unparalleled since many other compounds of a poisonous nature are likewise temporarily stored. That a toxic action is exerted by ammonium chloride may be concluded from the noticeably increased elimination of total nitrogen in the after-period of both dogs. The appearance of creatine in the urine only after the ammonium salt administration would tend perhaps to point in the same direction according to our present obscure understanding of the function of creatine. To this line of reasoning the objection may be raised that the length of the experiment would be sufficient to account for the increased total nitrogen output after the salt ingestion, *i.e.*, that animals maintained without food nitrogen for such a long period might well show an augmented urinary nitrogen output. Against such an argument may be placed the data in Tables 3 and 4 where the periods are fairly comparable with those in Tables 1 and 2 and yet in which no increased nitrogen excretion is to be observed. Finally, it may be urged that insufficient fuel value in the food was given since less than the calories recommended by Grafe and Schläpfer were supplied. In reply to this objection one needs only to refer to the work of Taylor and Ringer who have demonstrated that carbohydrate intake has little or nothing to do with the retention of the nitrogen of ammonium salts. This fact is also confirmed by the data presented in Tables 3 and 4.

TABLE 1.

Dog 1.

The daily diet consisted of 60 grams cornstarch, 60 grams sucrose, 60 grams lard, 10 grams bone ash and 600 cc. water. The total fuel value was equivalent to 80 calories per kilo at beginning of experiment when the dog weighed 13 kilos.

DATE 1912	BODY WEIGHT		URINE						CHLORINE INTAKE CALCULATED AS Cl	CHLORINE BALANCE PER DAY		NITROGEN IN FECES		REMARKS
	kilos	gram	Volume cc.	Specific gravity	Total N grams	Ammonia N mgms.	Creatinine mgms.	Creatine grams	Chlorine out- put calcu- lated as Cl	grams	grams	gram	grams	
July														
10	13.2	0	475	1.006	1.82	0.21	469	0	0.01	0		0.36	-2.18	Fore-period. Standard diet only.
11	13.2	0	510	1.007	1.68	0.21	434	0	0.02	0		0.36	-2.04	
12	13.0	0	560	1.005	1.50	0.19	440	0	0.01	0		0.36	-1.86	
13	13.0	0	550	1.008	1.42	0.20	432	0	0.01	0		0.36	-1.76	
Average	13.1	0	524	1.006	1.60	0.20	444	0	0.01	0		0.36	-1.96	Ammonium chloride pe- riod. In addi- tion to stand- ard diet 3.9 grams ammo- nium chloride = 1.0 gram N were fed per day.
14	13.0	1.0	550	1.010	2.46	0.41	432	0	1.16	2.59	+1.43	0.45	-1.91	
15	12.8	1.0	550	1.011	2.68	0.61	416	0	1.75	2.59	+0.84	0.45	-2.13	
16	12.7	1.0	655	1.008	2.40	0.78	392	0	2.42	2.59	+0.17	0.45	-1.85	
17	12.7	1.0	520	1.009	*(1.77)	(0.72)	(300)	0	(1.75)	(2.59)		(0.45)		
18	12.6	1.0	610	1.010	2.42	0.91	392	0	2.13	2.59	+0.46	0.45	-1.87	
Average (4 days)	12.7	1.0	591	1.010	2.49	0.68	408	0	1.86	2.59	+0.73	0.45	-1.94	
19	12.7	0	520	1.010	1.82	0.70	416	0	2.13	0	-2.13	0.37	-2.19	After-period. Standard diet only.
20	12.6	0	390	1.009	1.60	0.41	350	14	0.51	0	-0.51	0.37	-1.97	
21	12.6	0	445	1.010	1.79	0.33	385	21	0.17	0	-0.17	0.37	-2.16	
22	12.6	0	535	1.009	2.02	0.35	376	48	0.10	0	-0.10	0.37	-2.39	
Average	12.6	0	472	1.009	1.81	0.47	382	21	0.73	0	-0.73	0.37	-2.18	

* Some urine was lost on this day, hence excluded from balances and averages.

TABLE 2.

Dog 2.

The daily diet consisted of 85 grams cornstarch, 85 grams sucrose, 85 grams lard, 10 grams bone ash and 600 cc. water. The total fuel value was equivalent to approximately 80 calories per kilo at the beginning of the experiment when the dog weighed 18.0 kilos.

DATE 1912	BODY WEIGHT kilos	URINE					NITROGEN INTAKE grams	CHLORINE INTAKE CALCULATED AS Cl			CHLORINE BALANCE PER DAY grams	NITROGEN IN FECS grams	NITROGEN BALANCE PER DAY grams	REMARKS
		Volume cc.	Specific Gravity	Total N grams	Ammonia N grams	Creatinine mgms.		Chlorine out- put calcu- lated as Cl	grams	grams				
July														
10	18.6	0	1.010	1.78	0.32	011	0	0.0	0			0.55	-2.33	Fore-period. Stand- ard diet only.
11	18.5	0	1.010	1.57	0.31	568	0	0.10	0			0.55	-2.12	
12	18.5	0	1.010	1.60	0.33	500	0	0.10	0			0.55	-2.24	
13	18.4	0	1.010	1.70	0.32	500	0	0.20	0			0.55	-2.34	
Average	18.5	0	1.010	1.71	0.32	575	0					0.55	-2.26	Ammonium chloride period. In addi- tion to stand- ard diet 3.9 grams am- monium chloride = 1.0 gram N were fed per day.
14	18.2	1.0	1.010	2.64	0.57	576	0	1.16	2.50	+1.43		0.63	-2.27	
15	18.1	1.0	1.010	2.72	0.92	048	0	2.13	2.50	+0.46		0.63	-2.35	
16	18.2	1.0	1.010	2.04	1.14	584	0	2.52	2.50	+0.07		0.63	-2.27	
17	17.8	1.0	1.010	2.94	1.20	020	0	2.70	2.50	-0.20		0.63	-2.30	After-period. Stand- ard diet only.
18	17.5	1.0	1.010	2.81	(0.93)	(584)	(176)		(2.50)			0.63	-2.29	
Average	17.9	1.0	1.010	2.73	0.96	607		2.15	2.50	+0.44		0.63	-2.29	
(4 days)														
19	17.0	0	1.011	2.54	0.54	500	224	0	0			0.71	-3.25	After-period. Stand- ard diet only.
20	17.5	0	1.010	2.42	0.48	536	192	0.16	0			0.71	-3.13	
21	17.4	0	1.010	2.30	0.49	536	128	0	0			0.71	-3.01	
22	17.4	0	1.012	2.48	0.43	536	184	0	0			0.71	-3.19	
Average	17.4	0	1.011	2.43	0.58	542	182					0.71	-3.14	

* Animal vomited. N in vomit = 0.19 gram; urine contaminated with vomit.

In the absence of any more reasonable explanation it may be concluded that *ammonium chloride ingested by dogs maintained upon a non-nitrogenous diet is in part temporarily retained. This retention is accompanied by evidences of toxicity. Our experiments, therefore, fail to support the contention of Grafe and Schläpfer that the nitrogen of this salt may be utilized as a source of nitrogen supply. On the contrary, they furnish evidence that the ingestion of ammonium chloride may be regarded as a distinct detriment to nutritional rhythm.*

Experiments with ammonium acetate and citrate.

To determine whether in our hands other ammonium salts would yield results in harmony with those of Grafe and Schläpfer experiments have been carried out with ammonium salts of organic acids, namely, the acetate and the citrate.

Only a glance at Tables 3 and 4 is necessary in order to glean that these salts show a behavior totally unlike that yielded by ammonium chloride. These protocols give evidence of a considerable daily retention of nitrogen which fails to reappear during the after-period. There is no evidence of a toxic influence and indeed the figures for the after-period indicate a distinct sparing influence upon nitrogenous metabolism. Throughout the entire period in both instances creatine was always present in the urine. Its significance under these circumstances is not clear since the degree of its appearance in the two experiments is directly contradictory. Upon comparison of the effects of the two salts it would appear that considerably more nitrogen in the form of the citrate was retained than was true for the acetate. These experiments also emphasize that in any consideration of ammonium salts from the standpoint of intermediary metabolism a distinction must be made between salts of organic acids and those of inorganic acids. To again draw attention to this fact it is necessary only to point out the influence of a single day's ingestion of ammonium chloride (Table 4, October 23) upon the output of ammonia nitrogen. Ingestion of ammonium acetate or citrate has little or no effect upon ammonia nitrogen excretion. When, however, a comparable amount of nitrogen in the form of ammonium chloride is given the ammonia nitrogen elimination is mark-

TABLE 3.

Dog 3.

The daily diet was identical with that of Dog 1 in Table 1.

DATE 1912	BODY WEIGHT	NITROGEN INTAKE	URINE					NITROGEN IN FECS	NITROGEN BAL- ANCE PER DAY	REMARKS
			Volume	Specific Gravity	Total N	Ammonia N	Creatinine			
		grams	cc.		grams	gram	mgms.	gram	grams	
October										
22	13.0	0	350	1.012	2.17	0.22	270	0.34	-2.51	Fore-period.
23	12.6	0	370	1.015	2.41	0.22	261	0.34	-2.75	
24	12.6	0	680	1.008	2.03	0.19	275	0.31	-2.37	
Average		0	460	1.012	2.20	0.21	238	0.34	-2.54	
25	12.4	1.25	650	1.011	2.70	0.20	272	0.23	-1.68	10 grams of ammonium citrate = 1.25 grams N were fed per day.
26	12.4	1.25	560	1.015	2.71	0.21	260	0.23	-1.60	
27	12.4	1.25	600	1.010	2.03	0.19	264	0.23	-1.61	
Average		1.25	603	1.012	2.68	0.20	268	0.23	-1.66	
28	12.4	0	570	1.006	1.92	0.14	249	0.30	-2.20	After-period.
29	12.2	0	540	1.012	1.57	0.17	270	0.30	-1.87	
30	12.2	0	400	1.013	1.66	0.15	221	0.30	-1.90	
Average		0	503	1.010	1.72	0.15	247	0.30	-2.02	

TABLE 4

Dog 1.

The diet was identical with that detailed in Table 1.

DATE 1912	BODY WEIGHT	NITROGEN INTAKE	URINE					NITROGEN IN FEACES	NITROGEN BALANCE	REMARKS
			Volume	Specific Gravity	Total N	Ammonia N	Creatinine			
	kilos	grams	cc.		grams	gram	mgms.	gram	grams	
October										
11	13.6	0	580	1.010	1.92	0.19	378	64	-2.43	Fore-period.
12	13.6	0	515	1.010	2.02	0.18	372	114	-2.53	
13	13.2	0	545	1.010	1.56	0.14	384	113	-2.07	
Average	13.5	0	547	1.010	1.83	0.17	378	97	-2.34	
14	13.0	1.0	660	1.008	2.64	0.22	433	174	-1.96	5.64 grams ammonium acetate = 1.0 gram N were fed per day.
15	13.0	1.0	620	1.009	2.48	0.17	405	53	-1.80	
16	12.8	1.0	610	1.011	2.74	0.15	405	135	-2.06	
17	12.6	1.0	Urine contaminated with vomitus							
18	12.6	1.0	Urine contaminated with vomitus							
Average (3 days)	12.8	1.0	630	1.009	2.62	0.18	414	121	-1.94	
19	12.6	0	610	1.009	1.78	0.21	379	39	-2.14	After-period.
20	12.6	0	520	1.014	1.61	0.18	362	42	-1.97	
21	12.4	0	580	1.009	1.75	0.17	376	52	-2.11	
Average	12.5	0	570	1.011	1.74	0.19	372	44	-2.07	

TABLE 4.—(Continued).

TABLE 4.—(Continued).

DATE 1912	BODY WEIGHT kilos	NITROGEN INTAKE grams	URINE	NITROGEN IN FECES gram	NITROGEN BALANCE grams	REMARKS					
			Volume cc.	Specific Gravity	Total N grams	Ammonia gram	Creatinine				
							mgms.	mgms.			
October 22	12.4	0		urine	lost	0					
23	12.4	1.0	470	1.013	2.65	0.50		mgms. Chloride output as NaCl.			3.9 grams ammonium chloride = 1.0 gram N = 2.59 grams Cl were fed on this day.
24	12.2	0	280	1.013	1.68	0.20		0.50			
25	12.0	0	580	1.000	1.63	0.25		0.24			
26	12.0	0	490	1.011	1.50	0.19		0.09			
November 13	13.6	0	320	1.016	1.95	0.21		traco			
14	13.6	0	430	1.011	1.51	0.21		traco			4.3 grams NaCl = 2.60 grams Cl were fed on this day in 3 por- tions.
15	13.4	0	310	1.020	1.57	0.21		2.50			
16	13.2	0	470	1.014	1.53	0.26		0.60			
17	13.2	0	440	1.015	1.62	0.20		0.66			
18	13.0	0	280	1.020	1.78	0.22		0.20			

edly changed, showing a behavior quite similar to that in a dog upon an adequate mixed diet. The excretion of chlorine is also shown. For the sake of comparison an amount of sodium chloride containing approximately the same quantity of chlorine was administered also for a single day. Under these circumstances sodium chloride produced no influence upon nitrogen elimination and the excretion of chlorine was entirely comparable with that after the ingestion of ammonium chloride for one day. All of the ammonium salts tested showed a distinct tendency toward a diuretic action since in every instance the average urine volume of the experimental period was appreciably higher than in either of the other two periods.

CONCLUSIONS.

Contrary to the conclusions of Grafe and Schläpfer it is demonstrated that ammonium chloride shows an entirely different behavior from organic ammonium salts ingested by dogs maintained upon a high calorie non-nitrogenous diet. Ammonium chloride fed under the experimental conditions fails to diminish the loss of nitrogen from the body as determined in a fore-period without ammonium chloride addition; in other words, nitrogen was not retained in the sense of Grafe and Schläpfer.

Under these circumstances urinary ammonia nitrogen is markedly increased beyond the average normal output determined for the fore-period. This fact is regarded as indicative of a temporary retention of the salt which may be looked upon as exerting a marked toxic action. Such an influence is evident from the noticeably increased negative nitrogen balance of the after-period when no ammonium salts were added to the diet. The action of ammonium chloride in these experiments coincides with that previously observed in dogs kept upon an adequate mixed diet and also during a period of inanition.

It may therefore be concluded that ammonium chloride under the experimental conditions is incapable of acting as a source of nitrogen supply for the nutritional needs of the body.

A possible explanation for the discrepancy between the results of Grafe and Schläpfer and our own may perhaps be found in the critique of the methods employed by Grafe and Schläpfer.

Ammonium acetate and ammonium citrate fed under conditions identical with those obtaining for ammonium chloride markedly decrease the nitrogen loss of the experimental period, the average of the fore-period being employed as a standard. No evidence was obtained of the final excretion of the retained nitrogen during the after-period, nor was there any indication of an augmented urinary ammonia nitrogen excretion. Our results obtained with ammonium acetate and ammonium citrate corroborate those of previous investigators.

The present investigation again emphasizes that in any consideration of the influence of ammonium salts upon intermediary metabolism a distinction must be recognized between ammonium salts of organic acids and those of inorganic nature. The ability of the organism to dispose of these two types of salts is radically different.

COMMENTS ON THE COMMUNICATIONS OF FOLIN AND DENIS.¹

BY EMIL ABDERHALDEN.

(*From the Physiological Institute of the University of Halle.*)

(Received for publication, June 28, 1913.)

Folin and Denis have put forward a colorimetric method for the estimation of tyrosine. By the use of this method they have recorded yields of tyrosine which are higher than those hitherto obtained from proteins by any investigator. I have been able to show that tryptophane, hydroxytryptophane and hydroxyproline give the same color reaction as tyrosine, thereby proving that Folin and Denis were in error in stating that they had found a quantitative method for the estimation of tyrosine. Their values are much too high.

Folin and Denis have further shown that the non-coagulable nitrogen of the blood is increased when protein or the products of its hydrolysis are administered *per os*. Their most interesting and fundamental studies have been extended to different important problems. Against the conclusions of Folin and Denis we have raised the following objections: Folin and Denis have not proved that the non-coagulable nitrogen was actually in the form of amino-acids, nor have they in any way shown that the amino-acids are not united to form protein in the wall of the intestine.

In order to clearly separate facts from mere inferences Dr. Lampé and I wished first of all to determine whether compounds containing amino groups, but non-coagulable and giving no biuret reaction, were actually present in blood serum. We have furnished the proof of this. But in order to prove satisfactorily that digested protein reaches the blood solely in the form of amino-acids it is necessary to identify these amino-acids as such and estimate them individually.

¹ This *Journal*, xiv, pp. 453 and 457, 1913.

It is regrettable that Folin and Denis have attacked our criticism so vehemently and go so far as to assert that the hypothesis of protein regeneration in the intestinal wall has been recently modified simply for the purpose of retaining the hypothesis. This is not in accord with the facts. It has always been maintained that amino-acids must remain over and above those which undergo synthesis. Furthermore we have always emphasized that this working hypothesis should be abandoned as soon as facts are disclosed which are in opposition to it. I have no wish to take any part in polemical discussions of hypotheses; but Folin and Denis will doubtless agree with us that careful criticism of investigations has always been of value.

I have reluctantly referred to this matter since the form and tone of Folin and Denis's objections might wrongly give rise to the impressions that our criticisms were not made in the most amicable and impersonal spirit.

ON THE CEREBROSIDES OF THE BRAIN TISSUE.

SECOND PAPER.

By P. A. LEVENE.

(From the Rockefeller Institute for Medical Research, New York.)

(Received for publication, July 2, 1913.)

In a previous investigation on the "Cerebrosides of the Brain Tissue" Levene and Jacobs¹ have brought to light the following new facts: first, that cerebrosides differ not only in their behavior towards solvents, but also in their optical activity, and second, that fatty acids entering in the molecule of different cerebrosides are not identical. Namely, it was possible to isolate from the product of hydrolysis of mixed cerebrosides the *d*, *l*, and the *dl* forms of cerebronic acid. A small quantity of an acid analyzing for $C_{21}H_{43}O_2$ was also observed. However, it was not obtained in absolutely pure state, its quantity was small, and it was therefore regarded as an impurity.

The cerebrosides employed for last year's work were prepared by the method of Parcus. In this method of preparation fresh, not desiccated, brain pulp is boiled with an aqueous barium hydrate solution. Hence there was a possibility that various optical isomers of the cerebroside isolated in the course of last year's work were artificial products formed in course of preparation. Therefore, it was urgent to establish whether or not brain tissue extracted directly with indifferent solvents contained the same isomers.

In the course of the present year investigations were undertaken on the phosphatides of the brain, and in the process of preparation of this substance, there were obtained considerable quantities of cerebrosides. It was concluded to utilize this material for the investigation of the question whether or not the optical isomers of the cerebrosides found in the mixtures obtained by the method of Parcus were of primary or secondary origin.

¹ Levene and Jacobs: this *Journal*, xii, p. 389, 1912.

In the present year's work the material was obtained from desiccated brain tissue. The tissue was dried under diminished pressure, so that complete desiccation took place within very few hours. The dry material was extracted with hot 95 per cent alcohol, each extraction lasting not more than thirty minutes. The deposit formed on cooling of the alcoholic extract served for preparation of cerebrosides.

The material obtained in this manner differed considerably in its general appearance from that obtained by the method of Parcus. The material was separated in a considerable number of fractions, which differed in their solubility.

The fractions also differed in their optical activity. Most of the measurements were made in pyridine solution in a concentration of 6.66 per cent. Under these conditions the fractions were separated into optically active and inactive. The maximum rotation under the given conditions was $[\alpha]_D = +4.14^\circ$. The lowest rotation in pyridine solution was $[\alpha]_D = +3.05^\circ$.

On hydrolysis of the optically active fractions only cerebronic acid was obtained. It consisted, however, of a mixture of the optical isomers.

Since in last year's work the fatty acids were obtained by saponification of the esters by means of alkali it seemed possible that some racemization took place in course of saponification, and that originally the cerebrosides contain only one active acid. It was, therefore, concluded to test directly the optical activity of the esters obtained on hydrolysis of the cerebrosides. In fact it was this object that stimulated the work in the directions of thorough fractionation of the cerebrosides prior to hydrolysis.

It was found that in this direction the work of last year was substantiated, as there were obtained esters varying in their optical activity and having the composition of cerebronic acid ethyl ester. The highest rotation observed was $[\alpha]_D = +2.9^\circ$, the lowest was $[\alpha]_D = +1.6^\circ$. On the other hand, from the more soluble and optically inactive fractions of cerebrosides there were obtained also ethyl esters of cerebronic acid, which were optically inactive. Thus all these observations coincide with those of last year and seem to justify the conclusion arrived at in the previous communication. These conclusions seem still further justified by the following observation. When the cerebroside with a rotation

$[\alpha]_D = +4.14^\circ$, was dissolved according to Thierfelder in chloroform-methyl-alcohol mixture, then the rotation of a 3.6 per cent solution was $[\alpha]_D = +9.5^\circ$, and $[\alpha]_D = +10.7^\circ$ in a 6 per cent solution. Thierfelder's cerebrin had a rotation of $[\alpha]_D = +6.4^\circ$ — 8.4° in a 5 per cent solution, and on hydrolysis yielded only cerebronic acid. Furthermore Thierfelder's kersin gave on hydrolysis 25 per cent of the total fatty acid in form of cerebronic acid and yet it was optically inactive.

Notwithstanding all this, we are inclined to defer decision on the existence of optically isomeric cerebrosides containing cerebronic acid until more data on the subject are obtained. The reasons are the following: The more soluble fractions of the cerebrosides invariably contain an acid of the composition $C_{24}H_{43}O_2$. In this respect our observations fully coincide with the discovery of Thierfelder,² although the fractionation in our work was carried out by a process totally different from his. Further, two of our samples, which apparently contained only traces of cerebronic acid, and which were optically inactive in a pyridine solution, showed slight dextro-rotation in a Thierfelder's chloroform-methyl-alcohol solution at a temperature just below the boiling point of the solution. Furthermore it was observed that the specific rotation of the cerebrosides is considerably lowered by the lowering of the concentration of the tested solution. Hence, decision on the existence of isomeric cerebrosides of cerebronic acid has to be deferred until it will be possible to separate completely cerebrosides of cerebronic acid from the other cerebrosides. The new facts brought forward by the work of Thierfelder and by us carry no definite evidence either against or in favor of our last year's assumption.

Regarding the nature of the acid $C_{24}H_{43}O_2$ our observations differ from those of Thierfelder inasmuch as the melting point of the acid obtained by us, the melting point of its ethyl ester and of its lead salt seemed to coincide with those of lignoceric acid.

In this communication only the experiments dealing with the obtaining of the acid $C_{24}H_{43}O_2$ will be described in detail. The methods of fractionation and the properties of the cerebrosides will be communicated when individual cerebrosides are obtained in their final purity.

² Thierfelder: *Zeitschr. f. physiol. Chem.*, lxxxv, p. 35, 1913.

EXPERIMENTAL PART.

The cerebrine fraction was optically inactive and had the following composition:

0.1139 gram of substance gave on combustion 0.2955 gram of CO_2 , and 0.1187 gram of H_2O . $\text{C} = 70.75$; $\text{H} = 11.66$ per cent.

0.2000 gram of substance employed for Kjeldahl nitrogen estimation required for neutralization 2.45 cc. of $\frac{N}{10}$ acid. $\text{N} = 1.72$.

The substance contained neither phosphorus nor sulphur.

Ethyl ester of the fatty acid.

EXPERIMENT I. Thirty-six grams of the substance were taken up in 400 cc. of 98 per cent ethyl alcohol to which 20 cc. of sulphuric acid had been added. The mixture was boiled for seven hours with return condenser in a water bath. The reaction product was allowed to remain over night at room temperature of 25°C . In the morning, the solution was found filled with a mass of glittering scales. These were filtered off on suction. The residue was again taken up in 150 cc. of ethyl alcohol to which 5 cc. of sulphuric acid had been added and the heating repeated as previously for three hours. The reaction product was again allowed to cool at room temperature of 25°C . over night, and on the following day the mass of glittering scales was again filtered and dried in a desiccator. The weight of this substance was 2.5 grams.

The two mother liquors were combined and placed in a refrigerator at -1°C . over night. A second deposit separated in appearance similar to the first. This was again filtered on suction and dried in the desiccator. The weight of the second product was 2.8 grams. The mother liquor from the second deposit was cooled in an ice and alcohol freezing mixture. A gelatinous mass formed which was again filtered on suction and recrystallized out of about 10 cc. of acetone. The sediment formed in acetone was filtered, dried in a desiccator, and amounted to 0.1200 gram.

The first ester was recrystallized out of acetone and dried over night in a vacuum desiccator. It had a melting point of $56-57^\circ\text{C}$. and the following composition:

0.1116 gram of substance gave on combustion 0.3216 gram of CO_2 and 0.1294 gram of H_2O .

EXPERIMENT II. Twenty-five grams of cerebrine, prepared in the same manner as the substance employed in Experiment I, were taken up in 200 cc. of ethyl alcohol containing 10 cc. of sulphuric acid. This substance was treated in the same manner as in the first experiment. The total yield of the ester was 3 grams. It was not possible to obtain any other esters.

0.1168 gram of substance gave on combustion 0.3356 gram of CO_2 and 0.1398 gram of H_2O .

		Calculated for $\text{C}_{24}\text{H}_{40}\text{O}_7\text{C}_2\text{H}_5$:	Found:
Experiment I	{ C.....	78.8	78.59
	{ H.....	13.1	12.89
Experiment II	{ C.....	78.8	78.49
	{ H.....	13.1	13.42

A 10 per cent pyridine solution of the ester was found to be optically inactive.

Free acid.

In order to obtain the free acid the ester was saponified in the usual way by dissolving the ester in ethyl alcohol and adding an excess of aqueous sodium hydrate solution. The solution was boiled with return condenser on the water bath for four hours. The reaction product was poured into acetone, washed repeatedly with acetone, then suspended in ether, shaken vigorously for some time, filtered, and the operation repeated several times. The sodium salt obtained in this manner was decomposed with aqueous hydrochloric acid, and placed on the hot water bath until the free acid melted into an oil. The oil was allowed to solidify, the aqueous hydrochloric acid decanted and the free acid freed from hydrochloric acid by repeatedly suspending it in water, melting it on the water bath and allowing the oil to solidify. When the wash water was free from hydrochloric acid the free lignoceric acid was dissolved in a minimum quantity of absolute ethyl alcohol and the equivalent of lead acetate dissolved in methyl alcohol was added. A few drops of aqueous ammonia were then added to complete the precipitation of the lead salt. The mixture was allowed to cool, filtered on suction and washed repeatedly with absolute alcohol. The dry product was dissolved in hot toluene and hydrogen sulphide gas was passed through the solution. The filtrate from the lead sulphide was allowed to

remain in the refrigerator over night. The acid crystallized out. It was then filtered on suction and the last traces of toluene removed by melting the substance on an electric stove. The melting point of this acid was 81°C. Mixed with a sample of an acid obtained on oxidation of cerebronic acid the mixture gave the same melting point.

Molecular weight of the acid.

EXPERIMENT I. 0.9566 gram of the acid was dissolved in a mixture of benzin and absolute methyl alcohol and titrated by means of $\frac{N}{3}$ alkali. It required for neutralization 5.2 cc. of the acid.

EXPERIMENT II. 1.0626 grams of the substance required for neutralization 5.8 cc. $\frac{N}{3}$ alkali.

	Calculated for $C_{21}H_{40}O_7$:	Found:
M. W. (Experiment I).....	368	368
M. W. (Experiment II).....	368	366

The ethyl ester of lignoceric acid obtained from other fractions of the cerebrosides, which gave on hydrolysis not only lignoceric but also cerebronic acid, showed some irregularity. Thus the ester, when obtained in exactly the same manner as described in the first two experiments, even though it had been re-esterified twice out of acetone, possessed a melting point of between 60 and 62°C. However, when this ester was saponified and transformed into the free acid which had a melting point of 81°C. and a molecular weight of 36, and was again transformed into the ethyl ester, then the melting point came down to 56-57°C.

Lead salt of the acid.

The lead salt was prepared of the pure acid and recrystallized out of a solution consisting of equal parts by weight of benzene and toluene. The melting point of the substance was at 117.5°C

Comparison of the constants of lignoceric acid and of the acid obtained from the cerebroside.

	Lignoceric acid:	Acid from cerebroside:
Free acid, M.P.....	80-81°C.	81°C.
Lead salt, M.P.....	117°C.	117.5°C.
Ethyl ester, M.P.....	55°C.	56-57°C.

THE INFLUENCE OF PANCREATIC AND DUODENAL EXTRACTS ON THE GLYCOSURIA AND THE RESPIRATORY METABOLISM OF DEPANCREATIZED DOGS.

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(Received for publication, July 5, 1913.)

In their first contribution on the effects of the complete removal of the pancreas v. Mering and Minkowski¹ reached the conclusion that the diabetes which results can only be accounted for by the cessation of some function of this organ which is necessary for the utilization of sugar. "Utilization of sugar" (*Verbrauch des Zuckers*) in the adult organism means, sooner or later, complete oxidation of sugar. The ultimate proof that it is the ability of the organism to oxidize sugar and not the mere mobilization or availability of sugar which is lacking in the depancreatized animal is found in the two facts of low respiratory quotient and the complete elimination of ingested sugar which are now attested by a number of observers.²

Whether the capacity to oxidize sugar is *totally* lost as the result of this operation in dogs need not here detain us. The claim which has been based on the work of Porges and Solomon will be dealt with in a subsequent paper. The important facts are that sugar cannot, under the circumstances, be oxidized in sufficient quantity to be of any service, neither can it be stored (Minkowski); consequently it accumulates in the blood until the kidney barrier is overcome when it appears in the urine.

¹ *Arch. f. exp. Path. u. Pharm.*, xxvi, p. 384, 1889.

² Minkowski: *ibid.*, xxxi, p. 85, 1893; Mohr: *Zeitschr. f. exp. Path. u. Ther.*, iv, p. 910, 1907; Falta, Grote and Staehelin: *Hofmeister's Beiträge*, x, p. 199, 1907; Verzár: *Biochem. Zeitschr.*, xlv, p. 201, 1912; Hesse and Mohr: *Zeitschr. f. exp. Path. u. Ther.*, vi, p. 300, 1909.

The observation by Knowlton and Starling,³ confirmed already by McLean and Smedley,⁴ that the addition of a pancreatic extract to the blood of a depancreatized dog caused more sugar to disappear from the blood when it was perfused through the beating heart of the depancreatized dog has raised the hope that, at last, the essential pancreatic "hormone" has been discovered. This work cannot, however, be accepted as proving the restoration of pancreatic function until it is shown that the disappearance of sugar from the perfusion fluid denotes actual oxidation of sugar and not polymerization. The disappearance of sugar from a digest containing pancreatic and muscle extracts which Cohnheim⁵ reported was shown by Levene and Meyer⁶ to be due in reality to a condensation into a polysaccharide. Moreover, according to Elias,⁷ a solution of dextrose perfused through the liver of a turtle lost sugar when the fluid was made slightly alkaline and the liver stored glycogen. Knowlton and Starling thus far have excluded neither of these possibilities, the formation of a polysaccharide or the storage of glycogen in the heart muscle.

Loewi⁸ has indicated an altogether different type of explanation of Knowlton and Starling's results in his observation that a Locke's solution containing 0.02 per cent of KCl instead of 0.04 per cent caused, when perfused through a diabetic rabbit's heart, the disappearance of as much or more sugar from the fluid than when perfused through the normal heart. Loewi ascribes the effect to the K ion, and alludes to the possibility that sodium ions might drive out potassium.

The final test of such an hormone, however, will be the restoration of pancreatic function to the entire organism. The best criterion of such restoration is the respiratory quotient. Sugar might disappear from the urine and the excess sugar even from the blood after administration of such an extract and still might not be oxidized.

If its oxidation could be restored to normal, the hyperglycaemia should disappear following the disappearance of glycosuria.

³ *Journ. of Physiol.*, xlv, p. 146, 1912.

⁴ *Ibid.*, p. 470, 1913.

⁵ *Zeitschr. f. physiol. Chem.*, xlii, p. 401, 1904; xlvii, p. 253.

⁶ *This Journal*, ix, p. 97, 1911.

⁷ *Biochem. Zeitschr.*, xlviii, p. 120, 1912.

⁸ *Münch. med. Wochenschr.*, xiii, p. 690, 1913.

Before Starling and Knowlton's work appeared, the writers had planned some experiments on depancreatized animals with pancreatic and duodenal extracts, the point of departure being an observation by one of them (M.) a few years ago that a mixed glycerin extract of dog's pancreas and duodenal mucosa had, apparently, caused the sugar in the urine of a diabetic man, while he was maintained rigidly on the same diet of milk and eggs, to fall considerably. The observations could not at that time be continued and we had decided upon a series of experiments to test the possibility of a coöperative control of carbohydrate combustion by pancreas and duodenal mucosa.

After a few preliminary experiments with a depancreatized dog in a respiration apparatus constructed on the closed circuit plan of F. G. Benedict,⁹ it became apparent that we must study also the effects of the extracts on the glycosuria in order to know at what time we should expect an effect, if any, on the respiratory metabolism.

EFFECTS OF EXTRACTS OF PANCREAS AND DUODENUM ON THE D:N RATIO IN DEPANCREATIZED DOGS.

A. Effect of pancreas extracts.

At first we collected the urine in twenty-four-hour periods. Several such experiments were performed but we shall report only one for the reason that the results were largely negative.

EXPERIMENT I. *Dog I.* A large female pointer weighing 25 kgm. was completely depancreatized by Hédon's¹⁰ technique on March 8, 1912. On March 9-10 the dog had a D : N ratio of 3.26 with a body temperature of 38.4°. On March 11 an extract was made from 670 grams of cow's pancreas brought fresh from the abattoir, the fluid (Ringer's solution) being kept acid with HCl until after boiling. After filtration and neutralization with Na₂CO₃, one-half of the total filtrate (500 cc.) was injected subcutaneously in the evening and the other half the following morning.

⁹ *Amer. Journ. of Physiol.*, xxiv, p. 345, 1909.

¹⁰ *Arch. int. de physiol.*, x, p. 350, 1911.

TABLE I.

DATE March 1913	TIME	TOTAL N	TOTAL D	D : N	TEMP.	REMARKS
	p.m.	grams	grams		°C.	
9	1.00	18.98	62.00	3.26	38.4	Starvation.
10	2.20					
11	2.15					
		17.41	58.06	3.33	38.2	Injected 600 cc. of pancreatic extract subcutaneously in divided doses, made from 670 grams cow's pancreas.
12	2.20	15.06	57.80	3.83	39.8	One-half injected in evening of March 11. Remainder in morning of March 12.
13	2.20	14.78	52.82	3.57	38.4	Part of urine discarded on account of contamination.
14	2.20	7.33	21.80	2.97	38.4	
15	2.45	5.82	16.20	2.80	38.2	

The only effect which is noticeable in the first twenty-four hours is an increase in the D : N ratio, and it is evident that this is due to a greater fall in the output of nitrogen than in the output of sugar. There was, at the time of catheterization on March 12, a body temperature of 39.8°. The ratio continued high the next day but fell on March 14 and 15. A large abscess had by this time formed in one flank of the dog at the site of injection. This circumstance, although not accompanied by a rise in temperature, may have its bearing on the lower ratio since, as Minkowski¹¹ observed and as we ourselves have seen in a case of general peritonitis in one of our dogs, a serious infection with formation of pus prevents the appearance of sugar in the urine.¹² It is more likely that the ratios of 2.97 and 2.80 on the last days, however, simply represent an unusually late appearance of the typical ratio.

EXPERIMENT II. *Dog II.* Weight 12.3 kgm.; operated March 24, 1913; without food forty-eight hours before operation. Received no food through-

¹¹ *Arch. f. exp. Path. u. Pharm.*, xxxi, p. 85, 1893.

¹² Ehrlich long ago discovered that the leucocytes in diabetic blood contain glycogen and Levene and Meyer (this *Journal*, xv, p. 361, 1912), have recently demonstrated the ability of leucocytes to break up glucose *in vitro*.

out the experiment. Qualitative test for sugar immediately after operation. D : N ratio on subsequent day 2.85, temperature 39.2°.

On March 27 the ratio was 3.41 and on March 29, 3.19 in a two-hour period (Table II). Knowlton-Starling extract made from a dog's fresh pancreas was injected intravenously at 4.00-4.20 p.m. This dog, whose pancreas was used, had been fed 100 grams of dextrose one hour before removal of pancreas. The temperature was normal throughout the experiment.

TABLE II.

DATE March 1913	TIME	TOTAL N	TOTAL D	D PER HR.	N PER HR.	D FOR 24 HRS.	N FOR 24 HRS.	D : N PER HR.	D : N PER 24 HRS.	TEMP.
			grams	grams	grams	grams	grams			°C.
29	2.30 p.m. } 4.30 p.m. }	0.84	2.685	1.342	0.42	32.308	10.08	3.19	3.20	38.4
29	4.00 p.m. } 4.20 p.m. }	Entire extract of dog's pancreas injected by femoral vein. .								
29	4.30 p.m. } 6.30 p.m. }	1.922	2.937	1.468	0.961			1.52		
29	9.30 p.m.	1.400	4.20	1.400	0.466			3.00		38.2
30	12.30 a.m.	1.310	3.615	1.205	0.436			2.76		38.2
30	10.00 a.m. } 2.30 p.m. }	4.142	12.312	0.880	0.295	25.749	9.614		2.67	
31	2.30	10.44	30.00	1.250	0.420	30.00	10.44		2.97	38.4

The marked change in the D : N ratio in the period immediately following the injection is plainly due to a rise in the excretion of nitrogen. There is at the same time a smaller increase in the output of sugar which may be due to a washing-out process. In the third period, however, there is, without doubt, a decrease in the sugar which becomes more accentuated in the hourly excretion for the remainder of the twenty-four-hour period. The following twenty-four hours, with the excretion of nitrogen exactly the same as in the fore-period, the sugar returned almost to that level, making a normal Minkowski ratio. No influence of the extract, therefore, is clearly discernible in this experiment.

EXPERIMENT III. *Dog III.* Weight 7.8 kgm. Operated April 7, 1913. Without food for forty-eight hours before operation. D : N ratio on the second day following was 2.81. On April 8 a Knowlton-Starling extract was made from six cows' pancreases in 1000 cc. of Ringer's solution. 200 cc. of this extract were injected intravenously after making slightly alkaline with sodium carbonate.

TABLE III.

DATE April 1913	TIME	TOTAL N	TOTAL D	N PER HR.	D PER HR.	N FOR 24 HRS.	D FOR 24 HRS.	D : N PER HR.	D : N FOR 24 HRS.	TEMP.
	p.m.	grams	grams	grams	grams	grams	grams			°C.
8	3.40 } 7.20 }	1.12	3.40	0.33	0.927			2.81		38.5
8	6.10 } 7.20 }	200 cc. extract of cow's pancreas injected by femoral vein.								
8	7.20 } 11.40 }	1.19	3.125	0.274	0.721			2.62		38.2
8	11.40 }									
9	3.30 }	5.08	12.40	0.317	0.775	7.39	18.925	2.44	2.56	38.2
10	3.30	6.66	18.908	0.277	0.787	6.66	18.908	2.84	2.83	38.5

In a short period immediately preceding the injection in this instance, the hourly excretion of nitrogen was 0.33 gram and of sugar 0.927 gram. The D : N ratio was 2.81. The short period immediately following shows a change in the excretion of nitrogen to 0.274 and in the sugar to 0.721; D : N ratio, 2.62. For the remainder of the twenty-four-hour period, i.e., up to 3.30 the next day, the hourly excretion of dextrose rose slightly and that of nitrogen a little more, making a D : N ratio of 2.44. The following twenty-four hours the sugar remained constant but the nitrogen fell off, restoring the Minkowski ratio of 2.84.

The hourly excretion of sugar was lowest immediately following the injection and rose gradually until, on April 10-11, it was 0.810 gram (Table IV). The ratio on this day is low on account of a higher excretion of nitrogen for some unknown cause. The urine was clear of albumin.

In only one of the three experiments (III) is there any noteworthy effect of the extract of pancreas made by the Knowlton-Starling method on the elimination of sugar and in this case the typical D : N ratio was restored in the second twenty-four hours.

B. Effects of a mixed extract of pancreas and duodenal mucosa.

It is well known, from the experiments of Bayliss and Starling, that a substance (secretin) can be extracted from the duodenal mucosa in acid media which has a powerfully stimulating effect

on the external secretions of the pancreas. It seemed possible that a similar substance (or secretin itself) might be necessary to activate the pancreatic component which is responsible for the oxidation of sugar in the normal animal and that the two extracts acting simultaneously might restore this capacity to a depancreatized animal.

EXPERIMENT IV. The same dog was used as in Experiment III. The mixed extract was made in the same manner as before from the organs of a dog which one and one-half hours before anaesthetization had been fed 100 grams of dextrose. The entire extract contained in 150 cc. Ringer's solution was given intravenously on April 11, 5.00-6.00 p.m.

TABLE IV.

DATE April 1913	TIME	TOTAL N	TOTAL D	N PER HR.	D PER HR.	N FOR 24 HRS.	D FOR 24 HRS.	D:N PER HR.	D:N FOR 24 HRS.	TEMP.			
	p.m.	grams	grams	grams	grams	grams	grams			°C.			
10	3.30	7.89	19.460	0.328	0.810	7.89	19.460	2.46	2.46	38.5			
11	3.30												
11	5.00												
11	6.00	150 cc. extract of dog's pancreas and duodenum by femoral vein.											
11	3.30	6.72									1.413	0.268	0.565
11	6.30												
11	6.00	5.68	13.61	0.264	0.633			2.40	36.4				
12	3.30												

The double extract of pancreas and duodenal mucosa produced a greater effect on the hourly excretion of sugar than the extract of pancreas alone, notwithstanding the fact that a much greater weight of organ was extracted in the former experiment.

It was already apparent from the preceding experiment that the most marked effect of these extracts on the glycosuria is to be expected immediately after the injection. It was suggested also that when the sugar elimination for the twenty-four-hour period is reckoned up it might be found that the extract has caused only a temporary holding back of the sugar. Accordingly, in the next experiment, the urine was collected in short periods definitely delimited, as all our periods have been, by drawing the urine with the catheter and rinsing out the bladder with sterile water; and at the end the twenty-four-hour amounts were summed up.

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EXPERIMENT V. *Dog IV.* Weight 10.8 kgm. Operated April 17, 3.30 p.m. The D : N ratio on April 18, 2.15 p.m., was 2.60. On April 19 a Knowlton-Starling extract was made from the pancreas and duodenal mucosae of two dogs anaesthetized with chlorotone. The entire double extract was contained, after filtration, in 150 cc. Ringer's solution. No food was given at any time.

TABLE V.

DATE April 1913	TIME	TOTAL D	TOTAL N	D PER HOUR	N PER HOUR	D : N	D 24 HRS.
18	2.15 p.m.	25.87	10.08	0.932	0.363	2.56	21.048
19	6.00 p.m.						
19	6.00 p.m.	Injected	150 cc. of	pancreatic	and duodenal	extract	
	7.00 p.m.						
19	6.00 p.m.	1.33	0.672	0.605	0.305	1.98	
	8.12 p.m.						
19	10.12 p.m.	none	0.602	none	0.301		
20	12.23 a.m.	none	0.612	none	0.306		
20	6.00 p.m.	15.810	4.998	0.895	0.282	3.17	17.140
21	12.00 m.	21.730	8.442	1.20	0.469	2.57	
21	6.00 p.m.	6.107					27.837

Here the effect of injection was immediate and resulted in complete disappearance of sugar at the end of two hours from the time injection began or one hour from the time injection was discontinued. Sugar was completely absent for at least four hours and possibly longer. *Boiling with HCl did not reveal any reducing substance.* At 6.00 p.m. the next day, however, the amount of sugar per hour, reckoning from midnight when the last collection was made, had risen to the level of the fore-period.

The nitrogen after a preliminary fall from 0.363 to 0.305 remained constant for the full twenty-four hours, after which it rose with the sugar and the original ratio of excretion was resumed. The twenty-four-hour periods show a complete compensation.

EXPERIMENT VI. A second experiment identical in all respects with the first was performed on the same dog. The extract again was made from the pancreas and duodenal mucosae of two dogs.

TABLE VI.

DATE April 1913	TIME	TOTAL D	TOTAL N	D PER HOUR	N PER HOUR	D:N	BLOOD SUGAR	VOL- OF URINE
	p.m.	grams	grams	grams	gram		per cent	cc.
21	12.12 } 4.15 }	4.807	2.044	1.202	0.511	2.35	0.128	80
21	5.00 } 5.30 }	Injected 150 cc. of mixed extract intravenously.						
21	4.15 } 6.15 }	1.30	0.672	0.650	0.336	1.93		30
21	6.15 } 7.15 }	0.288	0.329	0.288	0.329	0.87		25
21	8.15	0.406	0.455	0.406	0.455	0.89	0.148	25
21	9.15	0.588	0.483	0.588	0.483	1.21		26
21	10.15	0.507	0.396	0.507	0.396	1.28		22
22	6.06	18.66	6.944	0.933	0.347	2.68		

The preliminary ratio in this experiment was not quite a typical one for a completely depancreatized dog although there can be no doubt that the organ was completely removed. The lower ratio was due to a high nitrogen figure. The absolute fall in the sugar elimination in this instance was even greater than in the previous experiment although the sugar did not completely disappear from the urine. The volume figures for the urine show that there was no diuretic effect. The blood was analyzed for sugar at 4.00 p.m., one hour before injection and at 8.15 p.m., nearly three hours after the injection ceased. The results show a rise from 0.128 to 0.148 per cent. Twenty-four hours after the injection dextrose and nitrogen were again being excreted in the typical ratio for complete pancreatic diabetes.

There are three possible explanations of the decreased elimination of sugar in these experiments; 1, that the permeability of the kidney to sugar has been reduced; 2, that glycogen has been temporarily stored; and 3, that sugar has been oxidized.

1. *Influence of pancreatic extracts on the permeability of the kidney.* De Meyer¹³ made the interesting discovery that when a pancreatic extract was added to Locke's solution perfused through an excised kidney, the permeability of the kidney to an excess of sugar in the perfusion fluid was diminished. About the same

¹³ *Arch. int. de physiol.*, viii, p. 121, 1909.

time Vahlen¹⁴ witnessed a decrease in the output of sugar when a preparation of pancreas was administered to phlorhizinized rabbits. G. Bayer¹⁵ made use of these facts in explanation of the effect of pancreatic extract on adrenalin glycosuria and Wohlgemuth¹⁶ invokes the same explanation to account for a hyperglycaemia without glycosuria which results from ligation of the pancreatic ducts. More recently, Scott¹⁷ injected subcutaneously a watery extract of pancreas into normal cats and obtained an increase in the blood sugar. To what constituent of the pancreas these effects are due is not yet known.

The increase in hyperglycaemia which went hand in hand with the reduction of the glycosuria in Experiment VI indicates that we are dealing here with an alteration of renal permeability.¹⁸ In order to account for the *amount of sugar* retained, however, it is necessary to assume either that some glycogen has been stored or that all the tissue fluids of the body contain sugar to the same extent (percentage) as the blood. The latter possibility would be very difficult to prove.

2. *Influence of the reaction of the medium on the storage of glycogen.* That the reaction of the medium may have a marked influence on glycogenesis and glycogenolysis has been established by the work of Macleod and by the recent work of Elias¹⁹ who found (a) a greatly reduced percentage of glycogen in the liver of normal rabbits, (b) considerable sugar in the urine of both rabbits and dogs, and (c) an active mobilization of glycogen from the liver of the turtle, on administration of dilute HCl. Conversely, there was an increase in glycogen in the turtle's liver when glucose contained in a dilute solution of sodium carbonate was perfused through it. In the liver of depancreatized turtles Nishi²⁰ had previously found a retention of glycogen upon perfusion with Ringer's solution containing glucose and de Meyer²¹ had reported the same for the liver of depancreatized dogs, *when pancreatic*

¹⁴ *Zeitschr. f. physiol. Chem.*, lix, p. 194, 1909.

¹⁵ Quoted by Biedl, *Innere Sekretion*, 1913, i, p. 500.

¹⁶ *Berl. klin. Wochenschr.*, 1913, Feb. 24, p. 339.

¹⁷ *Pr c. Soc. of Exp. Biol. and Med.*, x, p. 101, 1913.

¹⁸ Or possibly an effect on the colloid combination of sugar in the blood.

Cf. Allen: *Glycosuria and Diabetes*, Boston, 1913, p. 284, *et seq.*

¹⁹ *Biochem. Zeitschr.*, xlviii, p. 120, 1913.

²⁰ *Arch. f. exp. Path. u. Pharm.*, lxii, p. 170, 1910.

²¹ *Arch. int. de physiol.*, ix, p. 1, 1910.

extract was added to the perfusion fluid. Macleod and his co-workers, however, have been unable, under carefully controlled conditions, to prevent post-mortem glycogenolysis much less to cause glycogenesis in the perfused livers of warm-blooded animals and Macleod²² expresses utter disbelief in de Meyer's statement.

The following experiments were made for the purpose of testing the influence of weak alkali and weak acid on the glycosuria of the depancreatized dog.

EXPERIMENT VII. The same dog was used as in Experiment VI. Two days after that experiment Ringer's solution from the same bottle as that supplying the medium for the organ extracts was made alkaline to about the same degree (1 per cent) as the extract had been made, by adding Na_2CO_3 in substance and 150 cc. of this fluid were injected intravenously in exactly the same manner as in Experiment VI.

TABLE VII.

DATE April 1913	TIME	TOTAL D	TOTAL N	D PER HOUR	N PER HOUR	D:N	VOL. OF URINE					
		grams	gram	gram	gram		cc.					
24	2.19 p.m.	1.647	0.539	0.823	0.269	3.07	22					
	4.19 p.m.											
24	4.26 p.m.	150 cc. of 1 per cent Na ₂ CO ₃ intravenously.										
	5.15 p.m.											
24	4.19 p.m.	1.647	0.644	0.823	0.322	2.55	60					
	6.19 p.m.											
24	8.19 p.m.	0.466	0.524	0.233	0.262	0.88	24					
25	1.19 a.m.	2.413	1.475	0.480	0.295	1.62	90					

The dextrose elimination per hour and the D:N ratio reached the same level at the end of four hours as when the extracts were given.

Certain points of difference, however, may be noticed: 1, the effect is not immediate with the alkaline Ringer's solution alone; 2, the absolute fall in the sugar excretion is not so great; and 3, there is a marked diuretic effect with this solution while there was none with the organ extracts. These differences may prove to be significant, but, for the present, it will be assumed that they are not so important as the likeness of results between Experiments VI and VII.

EXPERIMENT VIII. Dog IV. Allowing two days to intervene, the same dog was next given an injection of 150 cc. of 2 per cent Na_2CO_3 . The

²² *Diabetes*, 1913, p. 124.

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osmotic effect of this solution would be similar to that of the 1 per cent Na_2CO_3 in Ringer's solution.

TABLE VIII.

TIME April 26, 1913	D PER HOUR	N PER HOUR.	D : N	VOL. OF URINE
	gram	gram	grams	cc.
10.45-12.45	0.527	0.227	2.31	19
1.30-2.15	150 cc. of 2 per cent Na_2CO_3 by femoral vein.			
12.45-2.50	0.280	0.301	0.93	120
2.50-4.50	0.191	0.231	0.82	34
4.50-6.50	0.535	0.233	2.30	33

The lower hourly excretion of sugar at the beginning of this experiment is the result of the gradual decline in sugar production which is always seen in progressive starvation following pancreatectomy. In the second two-hour period following infusion the elimination of sugar is even lower than in the previous experiment in which Ringer's solution was used. There is a primary rise in nitrogen elimination the first two hours, after which the same level is maintained as in the fore-period. The diuresis in the first after-period is much greater than in the previous experiment.

EXPERIMENT IX. *Dog V.* Female. Weight 12.1 kgm. Operated May 19, 1913. Dog had fasted for some days before operation. D : N ratio on May 20 at 9.15 a.m., was 2.32. Twenty-four hours later or forty-three hours after operation it was 2.94. At 11.25 on May 21, 200 cc. of a 2 per cent solution of HCl were given by stomach tube. None was regurgitated.

TABLE IX.

TABLE XX

TIME May 21, 1913	TOTAL D	TOTAL N	D : N	D PER HOUR	N PER HOUR	VOLUME
	grams	grams		grams	grams	cc.
Control specimen	33.8	11.53	2.94			
9.15 a.m. }	4.72	1.40	3.38	2.26	0.672	44
11.20 a.m. }						
11.25 a.m.	200 cc. 2 per cent HCl given by stomach tube.					
11.20 a.m. }	5.68	1.456	3.90	2.72	0.699	45
1.25 p.m. }						
1.25 p.m. }	5.68	1.41	4.03	2.84	0.705	40
3.25 p.m. }						
3.25 p.m. }	4.38	1.34	3.27	2.19	0.670	45
5.25 p.m. }						
5.25 p.m. }	33.3	11.03	3.02	1.85	0.613	
11.15 a.m.* }						

* May 22.

The effect of the HCl was immediate, and continued for four hours after which there was a compensating fall in the sugar elimination. Contrary to the effect of alkali, there was here no diuresis.

Both these experiments, therefore, turn out as would be expected on the hypothesis that mobilization of glycogen is favored by an acid medium and storage of glycogen by an alkaline medium. Final proof that it is an actual transformation of glycogen in the depancreatized dog as Elias has found it for the normal rabbit must wait on glycogen determinations.

In view of the directly opposite character of the results from the two experiments just presented, it is scarcely possible that the mere dilution of the blood with the infused fluid has produced any effect. Nevertheless, in order to leave no doubt on this point, we have controlled as follows:

EXPERIMENT X. *Dog VI.* Operated May 6. D : N ratio May 9, 3.08.

TABLE X.

TIME May 10, 1913	D PER HOUR	N PER HOUR	D : N
<i>p. m.</i>	<i>grams</i>	<i>gram</i>	<i>grams</i>
1.25-2.25	1.25	0.476	2.62
2.25-3.00	150 cc. sterile vein.	water introduced by femoral	
2.25-3.25	1.56	0.66	2.36
3.25-4.25	1.25	0.67	1.87
4.25-6.25	1.26	0.59	2.13

After a slight rise in the sugar excretion for the first hour, the hourly elimination is identical with that of the fore-period. The nitrogen elimination is higher after the infusion of water, probably owing to hemolysis; consequently the D : N ratio is lowered.

3. *The oxidation of sugar.* If the pancreas produces a substance the absence of which in the organism deprives the tissues of their power to oxidize dextrose, it should be possible to restore this substance either by extracting the pancreas or by utilizing blood from a normal animal (Hédon, 1892). There are many possible ways of extracting an organ and many of them have already been employed in an effort to isolate or at least to recover the essen-

tial "hormone" or internal secretion of the pancreas. Lépine,²³ Caparelli,²⁴ Ausset,²⁵ Vahlen;²⁶ Zuelzer,²⁷ E. L. Scott²⁸ and others have put forth claims for pancreatic preparations based, for the most part, on a reduction of the glycosuria either in diabetic persons or in depancreatized animals. None of these preparations, however, have been subjected to the essential test of restoring the power to oxidize sugar, and the facts brought to light by de Meyer, Wohlgemuth and Scott²⁹ regarding the effect of pancreatic substance on the permeability of the kidney or on the hyperglycaemia (which effects are most readily explained by such a renal action) have at once supplied an adequate explanation for any favorable effects on the glycosuria noted and have rendered very discouraging the quest for the true pancreatic "hormone." Knowlton and Starling's report that a substance soluble from fresh pancreas in acidified Ringer's solution had been found which enhanced if it did not fully restore the powers of the diabetic heart to "burn" sugar, came, therefore, as a very welcome announcement. The present writers confess to a feeling of disappointment that the "disappearance" of sugar from the perfusion fluid was not proved to be due to combustion. Instead of attempting to repeat Knowlton and Starling's experiments, however, it was hoped to find the evidence in an even more direct and crucial test, namely, by means of the respiratory quotient determined on the entire animal.

The time when the effect of the extract of pancreas and duodenal mucosa on the glycosuria was maximal having been found, the double extract³⁰ was injected intravenously into an animal which had been depancreatized two days before and whose D : N ratio and respiratory quotient both demonstrated its inability to burn sugar, and the respiratory quotient was followed beyond the point when the maximal effect on the glycosuria would have appeared.

²³ *Compt. rend. de soc. biol.*, cxiii, p. 1044, 1891.

²⁴ *Biol. Zentralbl.*, xii, Nos. 18, 19, 1892.

²⁵ *Semaine medicale*, xv, p. 326, 1825.

²⁶ *Loc. cit.*

²⁷ *Berl. klin. Wochenschr.*, xlvi, p. 1209, 1909.

²⁸ *Amer. Journ. of Physiol.*, xxix, p. 306, 1912.

²⁹ *Loc. cit.*

³⁰ The double extract rather than the extract of pancreas was used because of the greater effects on the glycosuria (see pages 371-373).

EXPERIMENT XI. Dog VI. Weight 12.1 kgm. Operated May 6, 1913. On May 9 the D : N ratio was 3.08. At 9.00 o'clock the animal was placed in the small calorimeter recently described by Williams²¹ and two one-hour periods were obtained with the dog perfectly quiet. The double extract was prepared from the organs of two dogs killed during this preliminary part of the experiment. After the preliminary experiment the dog was removed from the calorimeter and the entire extract of the glands and mucosae contained in 150 cc. of Ringer's solution made slightly alkaline after filtration, was injected by femoral vein. A urine period of an hour and ten minutes, including the injection period of twenty-five minutes, showed the immediate effect of the extract on the glycosuria. In order to make certain of the presence of sugar in the circulation, 20 grams of dextrose were then given by stomach tube, and the dog was returned to the calorimeter.

TABLE XI.

TIME May 9, 1913	CO ₂	O ₂	R. Q.	D : N	D PER HOUR	CAL. OF HEAT PRODUCED	BODY TEMP.
	grams	grams			grams		
9.45 a.m. }	8.62	9.22	0.68			21.73	38.6
10.45 a.m. }							
10.45 a.m. }	9.97	10.55	0.69	3.08	1.736	27.12	38.6
11.45 a.m. }							
12.35 p.m. }	150 cc. pancreatic and duodenal extract injected intravenously. 20 grams dextrose in 100 cc. H ₂ O per os.						
1.00 p.m. }							
11.55 a.m. }				2.70	1.068		
1.05 p.m. }							
1.50 a.m. }	13.60	14.13	0.70				39.6
2.50 p.m. }							
2.50 p.m. }	11.84	11.78	0.73				
3.50 p.m. }							
3.50 p.m. }	10.16	10.71	0.69		2.57		39.9
4.50 p.m. }							

It may be objected that a R. Q. taken three, four or even five hours after injection is only sufficient to prove that no sugar has been oxidized within that time and that it still remains possible that the pancreatic hormone would act later. In answer to this we may cite the compensating increase in the excretion of sugar which began between two and three hours after a similar injection in a previous experiment when no sugar was fed (page 373).

The slight rise in the R. Q. from 0.69 to 0.73 the second hour after infusion of the extract might be due to: 1, experimental error,

²¹ This *Journal*, xii, p. 317, 1912.

this variation being about the limit of error with the apparatus used; 2, the Na_2CO_3 given with the extract; or 3, the combustion of not more than 1 gram of dextrose in place of an isodynamic quantity of fat. Convincing proof that very little sugar at most could have been oxidized under the influence of the pancreatic substance injected in this experiment is found in the urinary ratios following. Twenty-four hours after the administration of the dextrose the D : N ratio was 2.62 (see page 377). The ratio just before administration was 3.08. Assuming that the ratio would have fallen uniformly if dextrose had not been given the mean output would have been 2.85 grams of dextrose for each gram of nitrogen. The total N for the twenty-four-hour period was 10.92 grams. The excess sugar, therefore, would be found by subtracting ($10.92 \times 2.85 =$) 31.12 grams from the total urinary sugar which was 50.32 grams. This accounts for 19.2 or all but 0.8 gram of the amount fed and proves again the complete compensation which takes place following the diminished glycosuria (page 372).

HEAT PRODUCTION OR TOTAL ENERGY METABOLISM OF THE DEPANCREATIZED DOG.

This dog had been used for calorimetric observations by Professor Graham Lusk one month previous to this experiment. At that time the basal metabolism was determined in three one-hour periods beginning eighteen hours after last food was given. The CO_2 elimination was 6.83 grams, the O_2 absorption 6.03 grams and the total heat production 18.37 calories per hour. In the experiment three days after pancreatectomy, when the D : N ratio was 3.08, the heat production had risen to 27.12 calories, an increase of 42 per cent. This agrees exactly with the result of Falta, Grote and Stachelin³² for a dog which bore a normal body temperature.

EFFECT OF NORMAL BLOOD ON THE R. Q. OF A DEPANCREATIZED ANIMAL.

Remembering the favorable effects on the glycosuria in the experiments of Forschbach³³ on two animals joined parabiotically

³² *Loc. cit.*

³³ *Deutsch. med. Wochenschr.*, xxxiv, p. 910, 1908; *Arch. f. exp. Path. u. Pharm.*, lx, p. 131, 1908.

and in those of Hédon³⁴ and of Drennan³⁵ on dogs transfused with normal dog's blood, the writers attempted also to demonstrate the combustion of sugar in a depancreatized dog which had received by direct anastomosis with a normal dog, 200 grams by weight, of the normal dog's blood, a corresponding amount of the diabetic blood having been previously drawn.

EXPERIMENT XII. *Dog VII.* Operated December 10, 1912. D:N ratio on December 12-13, 2.40.

TABLE XII.

TIME December 17, 1912	CO ₂	O ₂ *	R. Q.
<i>p. m.</i>	<i>grams</i>	<i>grams</i>	
1.15-2.15	5.66	6.32	0.651
2.18-3.20	5.65	5.82 (Note)	0.706
4.00	Transfused 200 grams normal dog's blood and fed 20 grams of glucose.		
5.00-6.00	6.43	6.35	0.726
6.00-7.06	5.83	6.18	0.685
8.30-9.30	6.25	6.77	0.672
9.30-10.30	5.55	5.62	0.718

* Variations in the total oxygen absorption are due to variations in the activity of the dog.

Six and one-half hours after transfusion no favorable effect had been observed. The amount of blood transfused was almost enough to cause the death of the donor, a dog of about 8 kgm. The transfusion of normal blood, in order to establish itself as a measure of any practical importance, should be expected to influence favorably the combustion of carbohydrate with much less than the total amount of "hormone" present in the circulation of an animal at any moment, *and to influence it immediately.* Had 1 gram of dextrose been oxidized in the first period after transfusion instead of an isodynamic quantity of fat the CO₂ output would have been increased by 0.41 gram which would have given a R. Q. of 0.78 instead of 0.73.

It is a singular fact that some observers who have reported the favorable effects on the glycosuria obtained by transfusion should not have taken account of the percentage content of sugar

³⁴ Latest work, *Arch. internat. de physiol.*, xiii, Heft 1, 1913.

³⁵ *Amer. Journ. of Physiol.*, xxviii, p. 396, 1911.

in the blood introduced. Sugar appears in the urine only when the hyperglycaemia reaches a certain height. To reduce this hyperglycaemia markedly by substitution of normal blood with its normal content of sugar must obviously reduce the glycosuria for a time, *without, however, necessarily influencing the combustion of sugar.*

• SUMMARY.

1. Intravenous infusion of pancreatic extract made from cow's pancreas by Knowlton and Starling's method raised the D : N ratio on the days immediately following, when the urine was collected in twenty-four-hour periods.

2. When the urine was collected in short periods a similar extract produced a slight fall in the hourly dextrose elimination and in the D : N ratio in the hours immediately following injection.

3. A mixed extract made in the same manner from dog's pancreas and duodenal mucosa produced a greater fall and in one instance complete disappearance of the urinary sugar. The fall, however, was followed in three to ten hours by a compensating increase.

4. A similar quantity of Ringer's solution made alkaline to about the same degree as the medium for the extract, produced an effect on the glycosuria almost identical.

5. A 2 per cent Na_2CO_3 solution likewise caused a sharp decline in the excretion of sugar and a 2 per cent HCl solution by stomach tube, produced a sharp increase. The increase in the one case continued for about the same length of time as the decrease in the other and both are probably to be explained by the effects of the medium on glycogenesis and glycogenolysis.³⁶

6. It is possible also that the pancreatic extract affected the renal permeability.

7. Neither extract of pancreas alone nor the double extract of pancreas and duodenal mucosa produced, within the time of maximal effect on the glycosuria, any effect on the respiratory quotient which could be interpreted as an index of increased combustion of carbohydrate.

³⁶ Cf. McLeod: *Diabetes*, 1913, pp. 150 and 183.

8. The total energy production of a depancreatized dog was found to be 42 per cent greater than that of the same dog while normal.

9. Transfusion of 200 grams of normal dog's blood likewise produced no observable effect on the R. Q. of a depancreatized dog.

We conclude that neither the use of the Knowlton-Starling extract nor the transfusion of normal blood is yet a measure of any practical importance in restoring to the depancreatized dog the ability to burn sugar.

PROTOZOAN PROTOPLASM AS AN INDICATOR OF PATHOLOGICAL CHANGES.

I. IN NEPHRITIS.

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Although pathological changes are undoubtedly in most instances the result of chemical disturbances, a simple biological method for demonstrating the existence of such changes has not been devised. The indication of subtle alterations occurring in one organism by, for example, the injection of an extract into another animal is obviously complicated by the highly specialized physiological processes of the vertebrate organism as well as by individual idiosyncrasies of the inoculated individual; and further, marked differences have been found in the toxic action of salts toward various tissues of the same animal, as well as toward the same tissue under different conditions. When, however, the organism is reduced to the lowest possible term, the single cell, these complications are considerably diminished if not entirely eliminated. Accordingly in the present work an attempt is made to demonstrate the existence of chemical changes arising from pathological conditions by determining the effects of normal and pathological tissue extracts on a unicellular animal, *Paramecium*.

Paramecium was adopted as our "biological reagent" because the ease with which it lends itself to experimental methods makes it one of the most favorable forms for general physiological study, and because we had at our disposal a pedigreed race of *Paramecium aurelia* which had been under daily observation for over six years or through more than 3700 generations.¹ This pedi-

¹ Woodruff: *Archiv für Protistenkunde*, xxi, p. 263, 1911; *Proc. Soc. Exp. Biol. and Med.*, ix, p. 121, 1912; *Biologische Centralblatt*, xxxiii, p. 34, 1913.

greed race afforded us an unfailing supply of protozoa whose environment and morphological² and physiological condition were accurately known for a long time and which was bred under particularly constant conditions during the period in which our experiments were made. The importance of these factors must be emphasized because numerous studies testify to the fact that the reactions of paramecia to various stimuli are greatly modified by their past and present environment.³ Greeley, for example, in a study of the effects of various chemicals on the protoplasm of *Paramecium* insisted that "maximum dilutions can only be approximate, as the action of identical solutions is not the same on paramecia from different cultures, because no two are exactly alike in respect to chemical composition and osmotic pressure."⁴ Further, since all the organisms we employed were derived originally from one animal, all the experiments were made on "sister" cells, and therefore on the "same protoplasm."

Previous experiments⁵ on this race of *Paramecium* have demonstrated that the rate of reproduction is a most accurate indication of the chemical composition and temperature of the environment, as remarkably slight variations in the culture medium produce characteristic responses by the animals. Indeed, the rate of reproduction may be said to be a function of the environment of the cell. Accordingly the present study involves a comparison of the rate of reproduction of subcultures from this pedigreed race of *Paramecium* bred on extracts of normal and of nephritic kidneys.

Methods.

1. *Conduction of cultures.* To initiate an experiment a single paramecium was isolated from the pedigreed race with a capillary pipette under a Zeiss binocular microscope and placed in a watch glass with a small amount of culture medium (hay infusion) similar to that employed for the main lines of the race. When, in the course of a few days, this animal by division had

² Woodruff: *Journ. of Morphology*, xxii, p. 223, 1911.

³ Woodruff: *Biol. Bull.*, xxii, p. 60, 1911.

⁴ Greeley: *Ibid.*, vii, p. 1, 1904.

⁵ Woodruff: *Biochem. Bull.*, i, p. 396, 1912; Woodruff and Bunzel: *Amer. Journ. of Physiol.*, xxv, p. 190, 1909; Woodruff and Baitsell: *Ibid.*, xxix, p. 147, 1911.

produced a sufficient number of cells for the experiment each of the organisms was isolated on a clean depression slide and supplied with five drops of the extract to be tested.⁶ Depression slides containing the eight lines of animals subjected to the same extract were placed in a moist chamber to prevent evaporation. At the start of the experiment and also at the time of the daily isolations precautions were taken to secure the inoculation with an identical bacterial flora of the extracts to be compared. The glassware employed was boiled in water redistilled from glass. All the organisms whose rate of reproduction was directly compared were bred at the same time and at the same room temperature. Consequently fluctuations in temperature affected all simultaneously and therefore need not be considered.

Each extract was tested on eight lines of sister cells for five days. Every day a single animal was isolated from each of the lines, placed on a clean depression slide and supplied with five drops of the extract from the test tube containing that day's supply. At the time of isolation a record was made of the number of divisions in each of the eight lines of cells during the previous twenty-four hours and these data are the basis of the results submitted in the present paper.

2. *Preparation of extracts.* In the preparation of kidney extracts extreme care was taken to exclude all possible sources of contamination. For the accomplishment of this object all vessels employed were of glass, usually of Jena make, and were used for no other purpose. These receptacles were cleaned with ordinary distilled water which had been redistilled in glass. Similar water was used in the preparation of the extracts. The technique employed in the preparation of the kidney extracts was as follows: The experimental animals were killed by bleeding, the kidneys immediately excised, dissected free from adhering fat, separately ground to a hash in a small meat chopper, and treated with a weight of water equal to five times the weight of the kidney. This mixture was slowly brought to the boiling point in an Erlenmeyer flask, covered with a watch glass to prevent undue evaporation, and maintained at this temperature for a period of five minutes with frequent shaking. The mixtures were then filtered.

⁶ Woodruff: *Journ. of Exp. Zoölogy*, x, p. 557, 1911.

In the preliminary experiments the filtration was made upon a folded filter, the solution being repeatedly passed through the paper to obtain a clear solution. In later work this was accomplished by filtering through a thick mat of washed filter pulp. In some instances neither type of filtration afforded more than an approximation to a perfectly clear solution. This was especially true for the extracts made from the nephritic kidneys. The filtered solutions for immediate use were placed in small test tubes, quantities sufficient for a day being employed. The surplus fluid was placed in small Erlenmeyer flasks. In either event the vessels were stoppered with cotton plugs, sterilized in an autoclave and kept in sealed jars in a dark, cool closet.

The behavior of Paramecium toward extracts of normal kidneys of the same and different animals.

It is obvious that the method of employing *Paramecium* as an indicator of pathological chemical changes must necessarily depend upon at least two conditions. In the first place it is conceivable that this organism may be so sensitive to slight chemical alterations as to render the method valueless; that, for instance, extracts of normal kidneys may vary sufficiently in composition to elicit a corresponding response on the part of *Paramecium*. On the other hand it is possible that, with the technique employed in the preparation of the extracts, the solutions finally used are not identical chemically. In other words, that two extracts prepared from normal kidneys of the same animal or of different animals will possess noticeably different composition.

To determine the degree of variability in the composition of such extracts as detected by *Paramecium* the following experiments have been carried through.

EXPERIMENT 1. The female rabbits, of approximately 2000 grams' weight each, selected for this experiment had been maintained under identical conditions for a period of at least three months and were in a state of good nutrition. The kidneys of Rabbit I each weighed 6.5 grams; those of Rabbit II, 5.0 grams. Extracts of each kidney made in the manner previously outlined showed a neutral reaction to litmus. These extracts were diluted with water in the ratio of one part of extract to three parts of water and were then employed as culture media for *Paramecium* of the pedigreed race already described, with the results recorded in Table I.

TABLE I.

The effects on the division rate of Paramecium of the extracts of normal kidneys of the same and of different rabbits.

	CULTURE A					CULTURE B					TOTAL DIVISIONS FOR BOTH CULTURES
LINE	NO. OF DIVISIONS AFTER DAYS					NO. OF DIVISIONS AFTER DAYS					
	1	2	3	4	5	1	2	3	4	5	

Rabbit I, Kidney A (normal).

1	1	5	8	11	15	3	5	7	11	15	
2	2	5	8	11	15	3	5	8	11	15	
3	2	5	8	10	14	2	5	7	10	14	
4	3	6	8	11	15	3	5	8	10	14	
Totals.....	8	21	32	43	59	11	20	30	42	58	117

Rabbit I, Kidney B (normal).

1	3	6	9	12	16	2	5	8	12	15	
2	3	6	8	12	15	3	6	9	12	16	
3	3	7	9	12	16	3	5	8	11	15	
4	2	5	8	12	15	2	4	7	11	15	
Totals.....	11	24	34	48	62	10	20	32	46	61	123

Rabbit II, Kidney A (normal).

1	3	6	9	12	17	3	6	8	11	15	
2	3	5	8	12	15	2	5	8	10	14	
3	3	6	9	12	16	2	5	8	11	14	
4	3	6	8	12	16	2	5	8	11	15	
Totals.....	12	23	34	48	64	9	21	32	43	58	122

Rabbit II, Kidney B (normal).

1	3	6	8	12	16	0	4	6	9	13	
2	2	6	8	11	15	3	6	8	11	15	
3	3	6	8	11	16	3	6	8	12	15	
4	3	6	8	12	15	3	6	8	11	15	
Totals.....	11	24	32	46	62	9	22	30	43	58	120

Inspection of the data will show that *Paramecium* fails to indicate any chemical difference in the extracts of the kidneys of the same animal or of different individuals as judged by the division rate of *Paramecium*, the variations occurring being within the limits of error of the experiment. It may therefore be concluded that these extracts possess a similar chemical composition, and further that the technique employed in their preparation is sufficiently accurate for the purposes of this investigation.

The influence of extracts of nephritic kidneys upon the division rate of Paramecium.

For testing the division rate of *Paramecium* toward pathological tissue extracts, use has been made of kidney extracts obtained from animals with tartrate nephritis. It has been shown⁷ that subcutaneous injection into rabbits of sodium tartrate causes marked histological changes in the convoluted tubules, accompanied by noticeable variations in the elimination of the urinary constituents. It is possible that these morphological changes are related to chemical alterations which may perhaps contribute directly to the almost invariable fatal outcome observed; although it has by no means been established that in nephritis toxic products are produced. At any rate this experimental condition affords an excellent opportunity to test the sensitiveness of our proposed biological indicator of chemical change.

During tartrate nephritis animals refuse food, hence it became necessary to test the influence of starvation alone as a control upon the reaction under discussion. This has been done in the experiment to be detailed below.

EXPERIMENT 2. Three female rabbits, kept under conditions identical with those of Experiment 1, were placed in metabolism cages. Rabbit III weighing 1900 grams, was subjected to simple starvation. Rabbits IV and V, each weighing 2500 grams, received two injections of 0.5 gram sodium tartrate respectively two days apart. No food was given. All three animals received a sufficiency of water. At the end of the third day the animals receiving the tartrate secreted the scanty colorless urine typical of nephritis and all the animals were killed by bleeding. Autopsy revealed the characteristic tartrate kidneys in Rabbits IV and V. The combined

⁷ Underhill: this *Journal*, xii, p. 115, 1912; Underhill, Wells and Goldschmidt: *Journ. of Exp. Med.*, xviii, Sept., 1913.

kidneys weighed as follows: Those of Rabbit III = 11.0 grams; those of Rabbit IV = 22 grams; those of Rabbit V = 25 grams. It will be observed that the nephritic kidneys were twice as heavy as those of the animal that had been subjected to starvation only. The softness of these tissues would lead one to infer that the increased weight was due to accumulated water. In order to make allowance for this possibility the extracts were suitably diluted, the details being given below.

In the preparation of the kidney extracts the organs of each animal were combined and treated in the manner noted under the section on methods. The extract obtained from Rabbit III was clear and definitely yellow in color. Those from Rabbits IV and V could not be filtered clear upon an ordinary paper and were quite turbid. The filtrates from the kidneys of Rabbits IV and V were divided into two portions, one of which was diluted with three times its volume of water, the other with one and one-half times its volume of water. The latter dilution was made on the assumption that the marked difference in weight in the kidneys noted above was due to water. The extracts were labeled as follows:

A = extract obtained from Rabbit III.

B = extract obtained from Rabbit IV.

C = extract obtained from Rabbit V.

TABLE II.

The effects upon the division rate of Paramecium of nephritic kidney extracts.

RABBIT	CULTURE	NO. OF DIVISIONS AFTER DAYS				
		1	2	3	4	5
I. Normal	A.....	4	17	32	45	53
	B.....	7	21	33	45	53
	—	—	—	—	—	—
	Total.....	11	38	65	90	106
III. Starvation, ext. A	A.....	8	20	33	46	53
	B.....	8	20	33	44	52
	—	—	—	—	—	—
	Total.....	16	40	66	90	105
IV. Nephritis, ext. B	A.....	8	15	30	42	49
	B.....	7	12	25	37	44
	—	—	—	—	—	—
	Total.....	15	27	55	79	93
V. Nephritis, ext. C	A.....	5	13	25	37	43
	B.....	5	15	27	39	45
	—	—	—	—	—	—
	Total.....	10	28	52	76	88

In each instance one part of the original extract was diluted with three times its volume of water.

The portions of the original extracts that were diluted with one and one-half times their volume of water were designated:

B' = extract obtained from Rabbit IV.

C' = extract obtained from Rabbit V.

In Tables II and III will be found the results obtained with these extracts when tested toward *Paramecium*. Table II contains the data furnished by the use of extracts A, B and C, when compared with an extract obtained from a normal kidney (Rabbit I, Experiment 1). The data in Table III were derived by the employment of extracts A (simple starvation), B' and C' and a normal kidney extract (Rabbit I, Experiment 1).

Comparison of the figures in Table II shows that: 1. The extract obtained from the kidney of an animal subjected to inanition for a period of three days does not differ from that of a normal kidney extract when tested toward *Paramecium*. 2. Nephritic

TABLE III.

The effect upon the division rate of Paramecium of nephritic kidney extracts.

RABBIT	CULTURE	NO. OF DIVISIONS AFTER DAYS				
		1	2	3	4	5
I. Normal	A.....	2	7	14	19	34
	B.....	1	8	13	15	32
		—	—	—	—	—
	Total.....	3	15	27	34	66
III. Starvation, ext. A	A.....	3	8	12	18	31
	B.....	3	9	15	19	29
		—	—	—	—	—
	Total.....	6	17	27	37	60
IV. Nephritis, ext. B'	A.....	0	3	6	13	26
	B.....	0	4	7	9	19
		—	—	—	—	—
	Total.....	0	7	13	22	45
V. Nephritis, ext. C'	A.....	0	0	3	5	17
	B.....	0	1	4	4	16
		—	—	—	—	—
	Total.....	0	1	7	9	33

kidney extracts cause a marked depression of the division rate of *Paramecium*.

On the assumption that the extra weight of the kidneys in Rabbits 4 and 5 was due to water, the comparative influence of extracts B' and C' was tested (see Table III). Inspection of the data here presented makes it evident that the depression of division rate noted in Table II cannot be ascribed to mere dilution, for the same type of action is even more markedly demonstrated in spite of the greater concentration of the pathological kidney extracts.

It may therefore be concluded that under our experimental conditions Paramecium is capable of distinguishing between normal kidney extracts and those obtained from kidneys rendered abnormal by sodium tartrate.

The behavior of Paramecium toward solutions of sodium tartrate.

There is a possibility that the depressing effect of nephritic kidney extracts may be explained by the presence in these extracts of significant quantities of tartrate itself. This suggestion appears very reasonable in view of the fact that tartaric acid is not readily burned in the organism and when subcutaneously administered is not eliminated to any extent. The specific action of tartrate upon the convoluted tubules also lends support to the assumption that a considerable portion of the injected tartrate may accumulate in the kidneys, and hence produce a depressing action upon *Paramecium* when the latter is placed in the kidney extract.

To determine the influence of various strengths of tartrate upon the division rate of *Paramecium*, pure recrystallized sodium tartrate was dissolved in a solution of beef extract.³ As a control beef extract without tartrate addition was employed.

From Tables IV and V it may be observed that strengths of tartrate below 0.1 per cent exerted little or no influence upon the rate of reproduction of the paramecia whereas 0.1 per cent solutions of tartrate caused a slight stimulation. The data in Table V demonstrate that sodium tartrate in 0.2 per cent solu-

³ Woodruff and Baitsell: Beef Extract as a Constant Culture Medium for *Paramecium*, *Journ. of Exp. Zoölogy*, xi, p. 135, 1911.

tion also has a stimulating effect, whereas in solutions of the strength of 0.3 per cent a marked depressant influence may be observed, resulting in death within five days. When tartrate solutions stronger than 0.3 per cent were employed the organism was quickly killed.

TABLE IV.

The effects upon the division rate of Paramecium of various strengths of sodium tartrate dissolved in beef extract.

SUBSTANCE TESTED	CULTURE	NO. OF DIVISIONS AFTER DAYS				
		1	2	3	4	5
Beef ext. (normal)	A.....	3	12	22	28	34
	B.....	2	7	19	23	26
		—	—	—	—	—
	Total.....	5	19	41	51	60
Beef ext. + tartrate (0.005 per cent)	A.....	3	11	22	29	36
	B.....	2	10	18	25	32
		—	—	—	—	—
	Total.....	5	21	40	54	68
Beef ext. + tartrate (0.001 per cent)	A.....	2	9	21	31	35
	B.....	3	6	18	26	32
		—	—	—	—	—
	Total.....	5	15	39	57	67
Beef ext. + tartrate (0.01 per cent)	A.....	3	8	18	23	30
	B.....	3	10	17	21	27
		—	—	—	—	—
	Total.....	6	18	35	44	57
Beef ext. + tartrate (0.03 per cent)	A.....	3	6	18	24	30
	B.....	2	6	19	28	33
		—	—	—	—	—
	Total.....	5	12	37	52	63
Beef ext. + tartrate (0.1 per cent)	A.....	2	8	22	31	41
	B.....	5	8	20	30	39
		—	—	—	—	—
	Total.....	7	16	42	61	80

The same type of test was applied to *Paramecium* employing normal kidney extract to which definite quantities of sodium tartrate had been added. In Table VI is shown the influence of

normal kidney extracts containing 0.025, 0.05, and 0.1 per cent sodium tartrate, and the conclusion may be drawn that tartrate in the strengths given above causes a slight stimulating action. It has been determined that from 0.2 per cent through 0.5 per cent sodium tartrate in normal kidney extract exerts a depressing influence. Comparison of this conclusion with that derived from comparable strengths of tartrate in beef extract shows that in the beef extract 0.1 and 0.2 per cent sodium tartrate has a stimulating influence. This behavior may be taken as a further proof

TABLE V.

The effects upon the division rate of Paramecium of various strengths of sodium tartrate dissolved in beef extract.

SUBSTANCE TESTED	CULTURE	NO. OF DIVISIONS AFTER DAYS				
		1	2	3	4	5
Beef ext. (normal)	A.....	4	7	8	9	12
	B.....	4	6	9	10	11
		—	—	—	—	—
	Total.....	8	13	17	19	23
Beef ext. + tartrate (0.2 per cent)	A.....	8	11	15	16	18
	B.....	7	12	16	17	23
		—	—	—	—	—
	Total.....	15	23	31	33	41
Beef ext. + tartrate (0.3 per cent)	A.....	5	8	10	10	
	B.....	6	10	10	10	
		—	—	—	—	—
	Total.....	11	18	20	20	Dead

of the sensitiveness of paramecia as an agent to detect chemical differences in their environment. When sodium tartrate in concentrations greater than 0.5 per cent was used the organisms were killed within forty-eight hours, and in some cases, for example with concentrations as high as 0.7, 0.8 and 0.9 per cent, in less than twenty-four hours.

From these observations it is clear that if the depressing effect of nephritic kidney extracts is to be ascribed to the presence of sodium tartrate, these solutions must contain the salt to the extent of at least 0.2 per cent. Such a possibility is exceedingly remote.

Nevertheless, in order to determine whether appreciable quantities of tartrate were present, tests for it were made by precipitation with calcium chloride. Preliminary trials with solutions of pure tartaric acid demonstrated that no precipitation occurred with calcium in solutions of less than 0.25 per cent. Extracts of the nephritic kidneys employed above failed to show any reaction with calcium. When these extracts were concentrated to one-

TABLE VI.

The effects upon the division rate of Paramecium of various strengths of sodium tartrate dissolved in normal kidney extract.

SUBSTANCE TESTED	CULTURE	NO. OF DIVISIONS AFTER DAYS				
		1	2	3	4	5
Kidney ext. (normal)	A.....	11	19	27	37	41
	B.....	12	18	27	37	41
	Total.....	23	37	54	74	82
Kidney ext. + tartrate (0.1 per cent)	A.....	13	21	31	43	47
	B.....	11	20	31	42	46
	Total.....	24	41	62	85	93
Kidney ext. + tartrate (0.05 per cent)	A.....	12	20	31	41	47
	B.....	12	21	31	43	46
	Total.....	24	41	62	84	93
Kidney ext. + tartrate (0.025 per cent)	A.....	13	20	30	40	43
	B.....	14	21	33	44	46
	Total.....	27	41	63	84	89

fifth their volumes the reaction was still negative. It is probable, therefore, that if tartrate was present in these extracts it must have been in a concentration less than 0.05 per cent tartaric acid. If this conclusion is correct it must be accepted that the depressing influence of nephritic extracts noted cannot be ascribed to tartrate, since concentrations of this salt in kidney extracts up to 0.1 per cent cause stimulation.

Although it may be granted that the presence of tartrate in the kidney extracts is not responsible for the depressant effect observed upon the rate of division of *Paramecium*, the problem has been attacked from another standpoint. It has been demonstrated that large doses of tartrate do not necessarily produce a more severe type of nephritis than small quantities;⁹ nevertheless it is reasonable to assume that, if tartrate does accumulate in the kidney, more would be present in extracts made from kidneys of animals receiving a large injection of tartrate than in kidney extracts prepared from rabbits to whom very small quantities of this salt has been administered. Under these circumstances it is evident that the extracts presumably containing the larger quantities of tartrate should exert a much greater influence upon the rate of division of the paramecia than might be anticipated from extracts with a small tartrate content. To determine the influence of extracts made under these conditions the observations noted below have been carried out.

EXPERIMENT 3. Five white female rabbits, each weighing approximately 1500 grams, and which had been kept for a reasonable period under identical conditions, were placed in metabolism cages. The animals were designated Rabbits VI, VII, VIII, IX and X. Rabbit VI was kept for a period of four days without food to serve as a control, no tartrate being administered. Rabbits VII and VIII each received 0.5 gram sodium tartrate after a two days' period of starvation, and under the same conditions 2.0 grams of sodium tartrate were administered to Rabbits IX and X. The preliminary two days' period of inanition was allowed in order to render the animals as sensitive as possible to the influence of tartrate.¹⁰ Sufficient water was given to each animal. In all four cases the typical symptoms—either colorless urine or anuria—prevailed at the end of the second day subsequent to tartrate injection. At this time the animals were killed by bleeding. The combined kidneys of each animal weighed as follows: Rabbit VI (normal) = 11.0 grams; Rabbit VII (very pale and soft) = 10.0 grams; Rabbit VIII (pale and soft) = 11.0 grams; Rabbit IX (pale and soft) = 15.0 grams; Rabbit X (very much congested) = 16.0 grams. Extracts of these kidneys were made in the usual manner. Considerable difficulty was experienced in preparing perfectly clear extracts but this object was finally attained by filtering through a heavy mat of filter pulp. It will be noted that kidneys of animals receiving the large dose of tartrate weighed considerably more than the others.

⁹ Underhill, Wells and Goldschmidt: *loc. cit.*

¹⁰ *Ibid.*

The influence of these solutions upon the division rate of *Paramecium* may be seen in Tables VII and VIII. These figures make it evident that if more tartrate does accumulate in the kidneys of animals receiving the larger dose of tartrate there is no evidence of

TABLE VII.

The effects upon the division rate of Paramecium of nephritic kidney extracts obtained from rabbits receiving small and large doses of sodium tartrate.

RABBIT	CULTURE	NO. OF DIVISIONS AFTER DAYS				
		1	2	3	4	5
VI. Normal (1:3)	A.....	8	17	28	37	47
	B.....	11	19	33	43	54
	—	—	—	—	—	—
	Total.....	19	36	61	80	101
VII. Nephritis, small dose tartrate (1:3)	B.....	8	14	23	31	39
	B.....	8	17	29	36	43
	—	—	—	—	—	—
	Total.....	16	31	52	67	82
VIII. Nephritis, small dose tartrate (1:3)	A.....	9	19	31	38	48
	B.....	9	17	30	38	45
	—	—	—	—	—	—
	Total.....	18	36	61	76	93
IX. Nephritis, large dose tartrate (1:3)	A.....	7	15	26	31	43
	B.....	8	15	27	33	43
	—	—	—	—	—	—
	Total.....	15	30	53	64	86
IX. Nephritis, large dose (1:2.25)	A.....	8	17	26	33	41
	B.....	6	14	26	31	40
	—	—	—	—	—	—
	Total.....	14	31	52	64	81
X. Nephritis, large dose (1:3)	A.....	8	15	27	35	46
	B.....	8	16	29	35	46
	—	—	—	—	—	—
	Total.....	16	31	56	70	92
X. Nephritis, large dose (1:2.25)	A.....	8	14	28	32	41
	B.....	7	14	26	31	42
	—	—	—	—	—	—
	Total.....	15	28	54	63	83

it indicated by the behavior of paramecia bred in such kidney extracts. At any rate paramecia do not show any different behavior when placed in kidney extracts of animals receiving large doses of tartrate than may be observed under similar conditions

TABLE VIII.

The effects upon the division rate of Paramecium of nephritic kidney extracts obtained from rabbits receiving small and large doses of sodium tartrate.

RABBIT	CULTURE	NO. OF DIVISIONS AFTER DAYS				
		1	2	3	4	5
VI. Normal (1:3)	A.....	10	20	30	41	50
	B.....	9	20	30	42	51
		—	—	—	—	—
	Total.....	19	40	60	83	101
VII. Nephritis, small dose tartrate (1:3)	A.....	5	12	19	26	34
	B.....	5	13	19	27	33
		—	—	—	—	—
	Total.....	10	25	38	53	67
VIII. Nephritis, small dose tartrate (1:3)	A.....	11	20	28	39	47
	B.....	8	17	27	37	45
		—	—	—	—	—
	Total.....	19	37	55	76	92
IX. Nephritis, large dose tartrate (1:3)	A.....	6	14	23	32	42
	B.....	4	12	22	30	39
		—	—	—	—	—
	Total.....	10	26	45	62	81
IX. Nephritis, large dose tartrate (1:2.25)	A.....	6	13	20	29	39
	B.....	7	14	21	29	37
		—	—	—	—	—
	Total.....	13	27	41	58	76
X. Nephritis, large dose tartrate (1:3)	A.....	5	14	22	33	42
	B.....	7	16	24	34	44
		—	—	—	—	—
	Total.....	12	30	46	67	86
X. Nephritis, large dose tartrate (1:2.25)	A.....	7	16	24	34	43
	B.....	4	14	21	30	39
		—	—	—	—	—
	Total.....	11	30	45	64	82

with kidney extracts of animals into whom only small quantities have been injected. It is therefore very improbable that the quantity of tartrate which perhaps may be present in kidney extracts of tartrate-injected animals plays any significant rôle in the depressant effect observed upon the division rate of *Paramecium*. One may therefore conclude that the *chemical changes underlying the pathological conditions in tartrate nephritis are responsible for the effect noted upon Paramecium. Conversely, Paramecium may be relied upon to give evidence of such chemical alterations under the conditions of our experiment.*

CONCLUSIONS.

Paramecium fails to indicate any essential difference in its division rate when subjected to the influence of extracts prepared from the separate kidneys of one rabbit or from kidneys of different individuals. Kidney extracts made from a starving rabbit behave in a manner identical with normal kidney extracts of well-fed animals.

The division rate of *Paramecium* is markedly depressed when placed in kidney extracts of rabbits with tartrate nephritis.

It has been demonstrated that this depressant influence cannot be associated primarily with tartrate which has accumulated in the kidneys, since quantities which could be present according to chemical tests would produce the opposite effect, namely slight stimulation of the division rate. Moreover, the depressant action of kidney extracts prepared from animals receiving large doses of tartrate is no greater than with extracts of nephritic kidneys of animals that had been given small doses of tartrate.

From these facts it is apparent that the pathological change—hence, presumably chemical alteration—in the renal tissue itself is responsible for the action observed upon the division rate of *Paramecium*.

Paramecium therefore may be regarded as a biological indicator of chemical change; and it is proposed to employ this biological method for the detection of chemical change under a variety of normal and pathological conditions.

PROTOZOAN PROTOPLASM AS AN INDICATOR OF PATHOLOGICAL CHANGES.

II. IN CARCINOMA.

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The detrimental influence of cancer growth upon the organism in general indicates that in the development of neoplasms abnormal metabolic conditions obtain. It is not improbable that under such circumstances there arise products from the altered metabolism which account in a measure for the observed symptoms. The extensive literature upon the subject fails to reveal positive evidence of the existence of such substances which may be held responsible for the clinical picture presented, although experiments on the influence of various products of normal metabolic activity on protozoa, seedlings, etc., have been carried out.¹

The importance of determining the presence of substances which may exert a detrimental action upon metabolic processes is obvious. We believe that the method outlined by us in a previous paper² for indicating chemical change is sufficiently reliable to demonstrate whether substances inimical to protoplasm are present in cancerous growths. We have therefore applied this method to the problem under discussion, employing primary carcinoma of the human breast as a characteristic type of abnormal growth.³

Methods.

In the cases here recorded the entire breast had been removed. Immediately after the operation extracts of uninvolved mammary tissue and carcinomatous tissue were prepared in exact accordance

¹ Calkins, Bullock and Rohdenburg: *Journ. of Inf. Dis.*, x, p. 421, 1912; Rosenbloom: *Biochem. Bull.*, ii, p. 229, 1912.

² Woodruff and Underhill: *this Journal*, xv, p. 385, 1913.

³ We are greatly indebted to Dr. William F. Verdi and Dr. Otto G. Ramsay for placing the tissues employed at our disposal.

with the directions previously outlined.⁴ Extreme precautions were taken to exclude all possible sources of contamination of the tissues. The rate of division of paramecia bred in the extract of the carcinoma has been compared with that in the extract of normal tissue of the same breast. The method of conducting the paramecia cultures is identical with that outlined in the preceding paper.⁵

*The effect of carcinoma extracts upon the division rate of Paramecium*⁶

In the preliminary experiments (Table I, Case 1) and Table II, Case 2) it is demonstrated that in the dilutions employed carcinoma extracts have little or no influence on the division rate of paramecia when compared with the growth of these organisms upon normal breast-tissue extracts. When, however, the original, undiluted extracts are employed an entirely different result is obtained (see Table III, Cases 1 and 2). Under these experimental conditions carcinoma extracts exert a primary depressant influence upon the division rate of *Paramecium* which invariably results in death within a period of from two to three days.

In Table IV, Case 3, are given the details of an experiment carried through a period of ten days. Inspection of these data will reveal several points of significance. In the first place it is evident that the original undiluted carcinoma extract has a marked depressant influence upon the rate of division of *Paramecium*, which is a direct confirmation of the results obtained in Cases 1 and 2. On the other hand, unlike those of Cases 1 and 2, the extract of Case 3 did not cause death in a single line of the culture. When the original extracts were diluted even very greatly, for instance 1:24, an effect was obtained just the opposite to that of the undiluted extract—a significant stimulation resulting.

From the figures given below (Table V) it is apparent that *Paramecium* is capable of distinguishing between the different concentrations of the same solution whether derived from normal or pathological tissue, thus again⁶ indicating the sensitiveness of

⁴ Woodruff and Underhill: *loc. cit.*

⁵ *Ibid.*

⁶ Woodruff and Bunzel: *Amer. Journ. of Physiol.*, xxv, p. 190, 1909.

TABLE I.

The effects upon the division rate of Paramecium of dilute solutions of carcinoma extracts.

Case 1.

SUBSTANCE TESTED	CULTURE	NO. OF DIVISIONS AFTER DAYS				
		1	2	3	4	5
Normal tissue Dilution = 1:3	A.....	3	17	28	41	49
	B.....	2	14	24	38	45
		—	—	—	—	—
	Total.....	5	31	52	79	94
Carcinoma Dilution = 1:3	A.....	4	16	24	38	47
	B.....	4	13	21	33	41
		—	—	—	—	—
	Total.....	8	29	45	71	88
Normal tissue Dilution = 1:6	A.....	3	15	24	39	44
	B.....	2	15	25	38	45
		—	—	—	—	—
	Total.....	5	30	49	77	89
Carcinoma Dilution = 1:6	A.....	6	19	26	34	41
	B.....	5	19	27	38	45
		—	—	—	—	—
	Total.....	11	38	53	72	86
Normal tissue Dilution = 1:12	A.....	3	14	21	33	40
	B.....	4	14	22	35	39
		—	—	—	—	—
	Total.....	7	28	43	68	79
Carcinoma Dilution = 1:12	A.....	4	14	23	35	41
	B.....	4	15	22	30	37
		—	—	—	—	—
	Total.....	8	29	45	65	78
Normal tissue..... Dilution = 1:24	A.....	4	13	16	31	35
	B.....	2	12	18	33	37
		—	—	—	—	—
	Total.....	6	25	34	64	72
Carcinoma Dilution = 1:24	A.....	5	10	16	26	29
	B.....	5	14	22	31	37
		—	—	—	—	—
	Total.....	10	24	38	57	66

TABLE II.

The effects upon the division rate of Paramecium of dilute solutions of carcinoma extracts.

Case 2.

SUBSTANCE TESTED	CULTURE	NO. OF DIVISIONS AFTER DAYS			
		1	2	3	4
Normal tissue Dilution = 1:3	A.....	8	16	29	42
	B.....	8	15	28	40
		—	—	—	—
	Total.....	16	31	57	82
Carcinoma Dilution = 1:3	A.....	7	15	29	39
	B.....	8	16	28	39
		—	—	—	—
	Total.....	15	31	57	78
Normal tissue Dilution = 1:6	A.....	8	17	29	41
	B.....	6	12	27	40
		—	—	—	—
	Total.....	14	29	56	81
Carcinoma Dilution = 1:6	A.....	8	16	29	42
	B.....	6	15	29	41
		—	—	—	—
	Total.....	14	31	58	83
Normal tissue Dilution = 1:12	A.....	8	14	29	36
	B.....	8	14	26	35
		—	—	—	—
	Total.....	16	28	55	71
Carcinoma Dilution = 1:12	A.....	8	13	22	33
	B.....	8	16	23	36
		—	—	—	—
	Total.....	16	29	45	69

TABLE III.

The effects upon the division rate of Paramecium of strong and weak extracts of carcinoma.

SUBSTANCE TESTED	CULTURE	CASE 1		CASE 2	
		NO. OF DIVISIONS AFTER DAYS			
		1	2	1	2
Normal tissue Undiluted	A.....	5	15	7	22
	B.....	5	15	8	22
		—	—	—	—
	Total.....	10	30	15	44
Carcinoma Undiluted	A.....	0	0	1	4
	B.....	0	0	2	6
		—	—	—	—
	Total.....	0	all dead	3	10 all dead
Normal tissue Dilution = 1:3	A.....	11	25	9	21
	B.....	10	24	10	21
		—	—	—	—
	Total.....	21	49	19	43
Carcinoma Dilution = 1:3	A.....	8	24	10	21
	B.....	8	23	9	20
		—	—	—	—
	Total.....	16	47	19	41
Normal tissue Dilution = 1:6	A.....	8	21	8	18
	B.....	9	21	8	18
		—	—	—	—
	Total.....	17	42	16	36
Carcinoma Dilution = 1:6	A.....	10	21	10	22
	B.....	8	21	8	20
		—	—	—	—
	Total.....	18	42	18	42

TABLE IV.

The effects on the division rate of Paramecium of strong and weak extracts of carcinoma.

Case 3.

SUBSTANCE TESTED	CULTURE	NO. OF DIVISIONS AFTER DAYS									
		1	2	3	4	5	6	7	8	9	10
Normal tissue Undiluted	A.....	5	17	30	38	49	58	71	81	89	103
	B.....	8	22	33	42	54	62	75	85	92	110
	Total.....	13	39	63	80	103	120	146	166	181	213
Carcinoma Undiluted	A.....	4	14	22	27	30	34	36	39	43	45
	B.....	5	14	22	27	32	35	37	39	42	45
	Total.....	9	28	44	54	62	69	73	78	85	90
Normal tissue Dilution = 1:3	A.....	6	18	29	36	47	56	69	76	84	102
	B.....	5	16	28	35	44	52	63	70	78	96
	Total.....	11	34	57	71	91	108	132	146	162	198
Carcinoma Dilution = 1:3	A.....	6	20	33	41	52	61	75	83	91	109
	B.....	8	22	35	40	52	62	76	84	92	110
	Total.....	14	42	68	81	104	123	151	167	183	219
Normal tissue Dilution = 1:6	A.....	4	15	25	27	35	42	51	60	64	78
	B.....	5	15	23	28	34	39	46	55	58	62
	Total.....	9	30	48	55	69	81	97	115	122	140
Carcinoma Dilution = 1:6	A.....	6	18	29	34	41	48	56	63	69	83
	B.....	4	19	30	35	42	49	58	68	76	95
	Total.....	10	37	59	69	83	97	114	131	145	178
Normal tissue Dilution = 1:12	A.....	5	13	17	21	25	29	36	42	48	59
	B.....	5	13	17	21	25	30	33	37	41	50
	Total.....	10	26	34	42	50	59	69	79	89	109
Carcinoma Dilution = 1:12	A.....	8	18	29	37	44	49	59	67	74	89
	B.....	6	15	27	31	40	46	51	59	66	76
	Total.....	14	33	56	68	84	95	110	126	140	165
Normal tissue Dilution = 1:24	A.....	3	9	13	14	17	18	20	22	24	26
	B.....	1	8	12	13	19	21	26	28	31	36
	Total.....	4	17	25	27	36	39	46	50	55	62
Carcinoma Dilution = 1:24	A.....	5	14	22	26	29	34	43	49	54	68
	B.....	6	17	25	28	31	35	40	45	50	52
	Total.....	11	31	47	54	60	69	83	94	104	120

this organism to quantitative chemical changes in its environment and therefore its value as an indicator of quantitative changes as well as of alterations presumably of a qualitative character.

The depressing influence of strong solutions of cancer extracts again finds corroboration in the data of Cases 4 and 5 contained in Tables VI and VII. Unlike those of Case 3, however, the weaker dilutions fail to reveal any stimulating influence upon the division rate of *Paramecium*. The detection of quantitative differences in the two solutions under discussion is again in evidence (see Table VIII) although the differences are not so sharply defined as in Case 3. In Cases 4 and 5, however, the strongest dilution of both solutions usually shows a tendency to inhibit the division rate of *Paramecium* when compared with extracts that have been diluted 1:3.

TABLE V.

Case 3.

DILUTION	DIVISIONS IN NORMAL EXTRACT		DIVISIONS IN CARCINOMATOUS EXTRACT	
	5 days	10 days	5 days	10 days
Undiluted	103	213	62	90
1:3	91	198	104	219
1:6	69	140	83	178
1:12	50	109	84	165
1:24	36	62	60	116

From a survey of the results of the five cases of carcinoma of the breast presented it is evident that in each instance the original undiluted extract of the abnormal tissue exerts a very pronounced depressant influence upon the division rate of *Paramecium* when bred under the conditions of our experiments. In certain instances this action may be so significant as to lead to death of the organisms within a comparatively short time. Weaker solutions of the abnormal extract may or may not show a stimulating action when tested toward *Paramecium*.

TABLE VI.

The effects upon the division rate of Paramecium of strong and weak extracts of carcinoma.

Case 4.

SUBSTANCE TESTED	CULTURE	NO. OF DIVISIONS AFTER DAYS				
		1	2	3	4	5
Normal tissue Undiluted	A.....	9	20	32	48	56
	B.....	<u>6</u>	<u>16</u>	<u>28</u>	<u>40</u>	<u>49</u>
	Total.....	15	36	60	88	105
Carcinoma Undiluted	A.....	4	13	20	29	35
	B.....	<u>4</u>	<u>13</u>	<u>22</u>	<u>32</u>	<u>36</u>
	Total.....	8	26	42	61	71
Normal tissue Dilution = 1:3	A.....	9	20	34	50	57
	B.....	<u>8</u>	<u>19</u>	<u>32</u>	<u>49</u>	<u>56</u>
	Total.....	17	39	66	99	113
Carcinoma Dilution = 1:3	A.....	7	19	31	47	53
	B.....	<u>9</u>	<u>18</u>	<u>32</u>	<u>49</u>	<u>54</u>
	Total.....	16	37	63	96	107
Normal tissue Dilution = 1:6	A.....	8	19	32	47	54
	B.....	<u>8</u>	<u>16</u>	<u>28</u>	<u>44</u>	<u>52</u>
	Total.....	16	35	60	91	106
Carcinoma Dilution = 1:6	A.....	9	17	29	45	51
	B.....	<u>8</u>	<u>18</u>	<u>31</u>	<u>47</u>	<u>52</u>
	Total.....	17	35	60	92	103
Normal tissue Dilution = 1:12	A.....	11	17	28	42	48
	B.....	<u>8</u>	<u>16</u>	<u>26</u>	<u>40</u>	<u>46</u>
	Total.....	19	33	54	82	94
Carcinoma Dilution = 1:12	A.....	9	14	27	41	48
	B.....	<u>10</u>	<u>16</u>	<u>29</u>	<u>44</u>	<u>50</u>
	Total.....	19	33	56	85	98
Normal tissue Dilution = 1:24	A.....	7	13	22	36	42
	B.....	<u>8</u>	<u>13</u>	<u>23</u>	<u>35</u>	<u>42</u>
	Total.....	15	26	45	71	84
Carcinoma Dilution = 1:24	A.....	8	15	25	36	40
	B.....	<u>7</u>	<u>12</u>	<u>24</u>	<u>36</u>	<u>40</u>
	Total.....	15	27	49	72	80

TABLE VII.

The effects upon the division rate of Paramecium of strong and weak extracts of carcinoma.

Case 5.

SUBSTANCE TESTED	CULTURE	NO. OF DIVISIONS AFTER DAYS				
		1	2	3	4	5
Normal tissue Undiluted	A.....	8	18	32	44	55
	B.....	5	17	30	40	50
	Total.....	13	35	62	84	105
Carcinoma Undiluted	A.....	2	5	7	12	15
	B.....	2	6	12	17	20
	Total.....	4	11	19	29	35
Normal tissue Dilution = 1:3	A.....	7	19	29	38	48
	B.....	6	18	30	39	48
	Total.....	13	37	59	77	96
Carcinoma Dilution = 1:3	A.....	7	18	31	39	49
	B.....	8	20	32	40	52
	Total.....	15	38	63	79	101
Normal tissue Dilution = 1:6	A.....	6	17	30	38	49
	B.....	6	20	30	39	48
	Total.....	12	37	60	77	97
Carcinoma Dilution = 1:6	A.....	5	16	29	38	47
	B.....	7	18	30	39	51
	Total.....	12	34	59	77	98
Normal tissue Dilution = 1:12	A.....	7	16	28	36	45
	B.....	7	17	29	35	44
	Total.....	14	33	57	71	89
Carcinoma Dilution = 1:12	A.....	6	14	26	33	42
	B.....	6	15	27	34	42
	Total.....	12	29	53	67	84
Normal tissue Dilution = 1:24	A.....	6	14	22	28	33
	B.....	5	13	21	29	34
	Total.....	11	27	43	57	67
Carcinoma Dilution = 1:24	A.....	6	13	24	29	33
	B.....	6	13	24	31	37
	Total.....	12	26	48	60	75

The influence upon Paramecium of extracts of normal and carcinomatous breast tissue of similar concentration.

In view of the sensitiveness of paramecia toward quantitative differences in the same solution it is possible that the influence of cancerous tissue extract may be accounted for entirely by assuming that this extract was merely quantitatively different from the normal extract. To test this hypothesis analyses were made of the extracts prepared from normal breast tissue and from the carcinoma in Cases 3, 4 and 5—total nitrogen, solids and ash being estimated (see Table IX).

TABLE VIII.

Case 4.

DILUTION	NORMAL	ABNORMAL
Undiluted	105 divisions	71 divisions
1:3	113 divisions	107 divisions
1:6	106 divisions	103 divisions
1:12	94 divisions	98 divisions
1:24	84 divisions	80 divisions

Case 5.

Undiluted	105 divisions	35 divisions
1:3	96 divisions	101 divisions
1:6	97 divisions	98 divisions
1:12	89 divisions	84 divisions
1:24	67 divisions	75 divisions

TABLE IX.

Composition of tissue extracts.

CASE	TISSUE EXTRACT	TOTAL N	SOLIDS	ASH
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
3	{ Normal.....	0.038	0.36	0.045
	{ Abnormal.....	0.060	0.63	0.073
4	{ Normal.....	0.050	0.43	0.047
	{ Abnormal.....	0.033	0.36	0.032
5	{ Normal.....	0.051	0.40	0.045
	{ Abnormal.....	0.039	0.34	0.030

These figures demonstrate that in Case 3 the greatest quantitative difference is to be found, the abnormal tissue extract being noticeably stronger than the normal breast extract. On the other hand the concentration of the normal extract in Cases 4 and 5 is considerably higher than the corresponding carcinoma extract. It is therefore apparent that mere quantitative differences in composition cannot be the sole cause for the effect upon *Paramecium* observed, since in the three cases the action of the original undiluted extracts was in the same direction, namely, marked depression of the division rate. In order, however, to further test this point the following experiments were carried out. Cases 3 and 4 were selected as typical examples—the abnormal extract in Case 3 being relatively much stronger than the normal extract; the normal extracts of Cases 4 and 5 being stronger than the cancerous extracts. Owing to lack of material in Case 5 it was deemed sufficient to take Case 4 as typical inasmuch as the two normal extracts of Cases 4 and 5 and the two extracts of abnormal tissue were almost identical in composition. Having selected these two cases as typical of the two tendencies in concentration of the substances determined, the stronger solution in each case was diluted with water in such a manner as to make it approximately equal in concentration to the weaker extract. The two solutions of each case, now nearly alike in concentration, were further diluted with water as in previous experiments and the resulting solutions were employed as culture media for *paramecia* with the results outlined in Tables X and XI.

It is at once apparent from these figures that the dilution of the stronger solution in each case did not affect the original characteristic action of these extracts upon the division rate of *Paramecium*. In other words, even when the two solutions in each case were made approximately alike in concentration as determined by the total solids, nitrogen and ash content, the marked depressant effect of the strong solutions may be observed upon the division rate of *Paramecium*. Likewise the general characteristics of the solutions are preserved. The stimulating action of the weaker solutions of Case 3 observed previously is again evident, whereas in Case 4 such an influence is inconstant—again in entire agreement with the results obtained with Case 4 outlined above.

TABLE X.

The effects upon the division rate of Paramecium of extracts of carcinoma made similar in concentration to extracts of normal mammary tissue.
Case 3.

SUBSTANCE TESTED	CULTURE	NO. OF DIVISIONS AFTER DAYS				
		1	2	3	4	5
Normal tissue Undiluted	A.....	8	20	34	43	53
	B.....	8	19	32	42	50
	Total.....	16	39	66	85	103
Carcinoma Undiluted	A.....	0	5	14	21	26
	B.....	0	5	13	18	24
	Total.....	0	10	27	39	50
Normal tissue Dilution = 1:3	A.....	7	15	26	32	39
	B.....	7	14	26	34	41
	Total.....	14	29	52	66	80
Carcinoma Dilution = 1:3	A.....	7	11	22	28	35
	B.....	6	13	21	29	35
	Total.....	13	24	43	57	70
Normal tissue Dilution = 1:6	A.....	8	12	19	23	28
	B.....	8	13	21	23	27
	Total.....	16	25	40	46	55
Carcinoma Dilution = 1:6	A.....	7	15	24	30	36
	B.....	6	14	23	29	33
	Total.....	13	29	47	59	69
Normal tissue Dilution = 1:12	A.....	5	11	16	21	22
	B.....	5	6	11	16	17
	Total.....	10	17	27	37	39
Carcinoma Dilution = 1:12	A.....	6	12	22	28	34
	B.....	6	12	23	29	33
	Total.....	12	24	45	57	67
Normal tissue Dilution = 1:24	A.....	5	9	16	18	18
	B.....	4	9	14	18	21
	Total.....	9	18	30	36	39
Carcinoma Dilution = 1:24	A.....	4	10	19	25	28
	B.....	4	10	13	19	21
	Total.....	8	20	32	44	49

TABLE XI.

The effects upon the division rate of Paramecium of extracts of carcinoma made similar in concentration to extracts of normal mammary tissue.

Case 4.

SUBSTANCE TESTED	CULTURE	NO. OF DIVISIONS AFTER DAYS				
		1	2	3	4	5
Normal tissue Undiluted	A.....	8	19	31	45	59
	B.....	8	19	31	45	57
	Total.....	16	38	62	90	116
Carcinoma Undiluted	A.....	1	3	4	4	4
	B.....	1	4	7	10	11
	Total.....	2	7	11	14	15
Normal tissue Dilution = 1:3	A.....	8	14	28	40	52
	B.....	9	19	31	44	59
	Total.....	17	33	59	84	111
Carcinoma Dilution = 1:3	A.....	10	19	28	40	54
	B.....	10	18	30	41	53
	Total.....	20	37	58	81	107
Normal tissue Dilution = 1:6	A.....	10	14	24	37	47
	B.....	8	12	22	34	45
	Total.....	18	26	46	71	92
Carcinoma Dilution = 1:6	A.....	9	16	26	38	49
	B.....	9	16	28	40	52
	Total.....	18	32	54	78	101
Normal tissue Dilution = 1:12	A.....	7	14	22	34	44
	B.....	5	11	19	31	39
	Total.....	12	25	41	65	83
Carcinoma Dilution = 1:12	A.....	5	9	18	28	37
	B.....	7	11	18	30	40
	Total.....	12	20	36	58	77
Normal tissue Dilution = 1:24	A.....	6	9	16	24	32
	B.....	6	8	16	24	33
	Total.....	12	17	32	48	65
Carcinoma Dilution = 1:24	A.....	4	8	17	25	35
	B.....	5	10	17	25	33
	Total.....	9	18	34	50	68

The effect of carcinoma extracts upon the division rate of *Paramecium* may be due to the absence or to a deficiency in these solutions of substances essential for the life processes of *Paramecium*, or to the presence in these extracts of substances inimical to protoplasm. If the latter hypothesis is correct it is possible, in view of the probable greater concentration of these substances in the neoplasm, that their absorption may contribute to the production of the symptoms characteristic of the development of cancerous growths. Further work on cancerous tissues is in progress tending toward the solution of some of the obvious problems suggested by our results.

CONCLUSIONS.

In certain concentrations extracts of carcinoma of the breast show a very pronounced depressant influence upon the division rate of Paramecium when compared to that obtained with Paramecium bred in normal mammary tissue extract. In some instances the depressant influence may be so profound as to lead to the death of the paramacia within two or three days.

Weaker dilutions of the abnormal tissue extracts may show a stimulating action upon *Paramecium*.

It has been demonstrated that the difference in concentration which may exist between the normal and abnormal breast tissue extracts cannot be held responsible for the detrimental action of the abnormal extract upon *Paramecium*, for when the concentrations of the two extracts under discussion are made as nearly equal as possible the original type of action still is observed.

ON THE FORMATION OF HYDROCYANIC ACID FROM PROTEINS.

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The question arose during the progress of a recent criminal trial whether decomposing proteins could, under any condition, liberate hydrocyanic acid. A search of the literature available failed to reveal any instances recorded. A great many experiments were therefore started in order to determine the question.

The method of testing for hydrocyanic acid was the Schönbein test with the modifications as suggested by Dr. Walter S. Haines, which are as follows:

A strip of fresh ash-free Swedish filter paper, about 8 cm. long and 0.5 cm. wide, is dipped about one-third of its length into a freshly prepared 10 per cent tincture of guaiac, then withdrawn and held upright in the air for a few moments until some of the alcohol has evaporated; then a drop of aqueous copper sulphate, one to one-thousand (1-1000) was placed on the tip of the filter paper so that it moistens not more than half of that which has been wet with the tincture of guaiac. The paper thus prepared is suspended above the solution to be tested for hydrocyanic acid for one minute and if hydrocyanic acid is present, even in dilutions as great as 1 part to 3,000,000, it will color distinctly blue that part of the paper only which has been wet with copper sulphate. If a volatile oxidizing agent is present, it will color blue, not only the tip which has been moistened with both copper sulphate and tincture of guaiac, but also that part which has been moistened with the tincture of guaiac.

The test as applied in this manner affords at the same time then a good way of distinguishing between hydrocyanic acid and volatile oxidizing substances. Ammonia, when it is present, will turn the entire paper a bluish green if dilute and a yellowish brown color if more concentrated. The presence of ammonia can be prevented by keeping the solution slightly acid. Hydrogen sulphide, if it is present, interferes with the test and if a guaiac-copper sulphate paper which has been first turned blue by hydrocyanic acid fumes is placed in hydrogen sulphide, the hydrogen sulphide will bleach out the blue color quite rapidly.

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Eggs were used in the first experiments: They were broken and the whites separated from the yolk and distributed into wide mouthed 500 cc. flasks so that each flask contained either the whites or the yolks of two eggs. The flasks were loosely stoppered and put in a warm place and tested each day by the Schönbein test for hydrocyanic acid.

Seventy-four experiments were started in nine different series and in eight experiments from six different series, after intervals varying from four days to fourteen days, the egg substance evolved hydrocyanic acid. In one case the egg substance continued to give off hydrocyanic acid for thirty-six days after which time it had all been used up inoculating other protein media. The eggs which gave the test best remained acid to litmus and phenolphthalein, but, at no time, were acid to dimethyl amido-azobenzol. They did not become putrid like those which decomposed without giving off hydrocyanic acid. We have good reasons for believing that a number of the eggs which in decomposing became putrid, also gave off hydrocyanic acid, but the hydrocyanic acid escaped detection as the hydrogen sulphide interfered with the test used. In a number of such cases, we obtained crystals which looked like silver cyanide although the silver nitrate was darkened by the hydrogen sulphide, but we were not able to get positive results with the Prussian blue test.

The yolks developed hydrocyanic acid in six cases and the whites in two cases and inoculation experiments showed egg yolk to be a more favorable medium than egg white.

That it was hydrocyanic acid that was given off was confirmed by obtaining the characteristic silver cyanide crystals, then decomposing these crystals with sodium hydroxide, ferrous sulphate and ferric chloride and subsequent acidulating with hydrochloric acid and obtaining the Prussian blue color. In some cases, the odor of hydrocyanic acid was easily detected when the stopper to the flask was first removed. That the reaction was not due to sulphocyanic acid was shown by dipping some filter paper in ferric chloride solution and suspending this over the liquid in the flask and the paper did not turn red as it does in the presence of sulphocyanic acid.

That the decomposition of this protein was due to some micro-organism was indicated by the following facts. A great many

inoculations were made from solutions that were giving off hydrocyanic acid abundantly, into fresh media and in every case, after incubation for twenty-four hours, they gave good tests for hydrocyanic acid. The organism was killed by the presence of free mineral acid, and also was destroyed by heating.

Egg culture 41, was, therefore, given to Messrs. Clawson and Young of the Department of Bacteriology to determine for us the organism which, in living on protein media, gave off hydrocyanic acid. They determined it to be *Bacillus pyocyaneus*.¹

The organism gives off hydrocyanic acid in larger quantities when grown on yolk than when grown on white. It is a good hydrocyanic acid producer when grown on gelatin, which it liquefies. It will produce hydrocyanic acid when grown on milk, but not abundantly, and when grown on liver tissue.

The organism grows best, or rather gives off hydrocyanic acid more rapidly when grown in a medium that is slightly acid to litmus and phenolphthalein. Repeated trials showed that the organism does not develop hydrocyanic acid at all when placed in eggs to which just sufficient hydrochloric acid has been added to give a test for free mineral acid.

When there is added to a culture, that is giving a good test for hydrocyanic acid, sufficient 2 per cent hydrochloric acid to just give a test for free mineral acid and sufficient time allowed for the hydrocyanic acid, which had been developed before, to disappear, the culture will no longer develop hydrocyanic acid. And if new media is now inoculated with this solution, it will not give off hydrocyanic acid which indicates that the presence of free mineral acid kills the organism.

The experiments are being continued and will be extended to other organisms.

CONCLUSIONS.

1. Certain microorganisms living on protein media evolve hydrocyanic acid.
2. These organisms liberate hydrocyanic best when living on a protein media slightly acid to litmus and phenolphthalein.
3. They do not liberate hydrocyanic acid in media containing free mineral acid.

¹ See this *Journal*, xv, p. 419, 1913.

PRELIMINARY REPORT ON THE PRODUCTION OF HYDROCYANIC ACID BY BACTERIA.

By B. J. CLAWSON AND C. C. YOUNG.

(From the Department of Bacteriology, University of Kansas.)

(Received for publication, June 28, 1913.)

Egg culture 41 which had produced HCN spontaneously, and which is mentioned in the paper by Emerson, Cady, and Bailey¹ was turned over to us by Dr. Cady for investigation to determine the organism, if any, which caused the generation of hydrocyanic acid. The modified Schönbein test was used throughout this work to detect the presence of hydrocyanic acid gas.

Stained smears were made of the original material in which an almost pure culture of a short rod was seen. Transfers of this material to sterilized whole egg developed hydrocyanic acid in twenty-four hours. From these tubes, the material was plated out on agar and grown at room temperature. After forty-eight hours, colonies appeared which produced a blue-green pigment that was distributed through the agar. Transfers to gelatin showed rapid liquefaction at room temperature. After forty-eight hours, the presence of HCN gas was revealed by the Schönbein test and confirmed by the Prussian blue reaction.

The principle characteristics of the organism are as follows: short rod; no spores; slightly pointed at the ends; decidedly motile; Gram negative; liquefies gelatin rapidly at 22°; brown growth on potato; peptonizes milk which becomes slightly alkaline; reduces nitrates to nitrites and ammonia; produces indol; no fermentation or production of gas in lactose, dextrose, saccharose, raffinose, salicin, inulin, mannite, or dulcete broth media. It does not reduce neutral red; forms heavy pellicle on broth, and green fluorescent pigment containing pyocyanin on agar. It will not grow in media which are even slightly acid to sodium alizarin sulphonate.

¹ This *Journal*, xv, p. 415, 1913.

420 Bacterial Production of Hydrocyanic Acid

From the above we conclude that the organism is *Bacillus pyocyaneus*. No attempt was made in this work to distinguish between *Bacillus pyocyaneus* and *Bacillus fluorescens* as both have the HCN-producing power.

The organism was grown on 50 grams of gelatin in an Erlenmeyer flask at room temperature. After growing for twenty-four hours and testing for HCN production, the flask was connected with a U tube containing 2 cc. of a 10 per cent solution of silver nitrate. The air that filtered through the cotton plug was drawn slowly through the flask and U tube for seventy-two hours. The nitrate and precipitate were then transferred to a small distilling flask and strong HCl added. Five cubic centimeters were distilled off into a receiver buried in freezing mixture. Of this distillate 0.2 cc. killed a chick in less than thirty seconds; 0.4 cc. given to a three-weeks' old kitten, killed it almost instantly.

The power which the organism has of producing HCN, is apparently not due to an extracellular enzyme. The organism was grown in gelatin for seventy-two hours at 37°, and had been giving off HCN for forty-eight hours. After filtering through a Berkefeld filter, some of the filtrate was planted again into gelatin and incubated, but gave negative results for HCN.

Hydrocyanic acid gas is apparently produced only under aerobic conditions, which led to the belief that the reaction in which HCN was produced was due to oxidation of the proteins. This was subsequently shown to be true. While good tests were always obtained from cultures grown at room temperature (22°), as a rule cultures grown at 37° gave a stronger test for HCN. Different media were used to determine whether or not the ability to produce HCN would be shown in them. Positive tests were obtained at 37° from gelatin, broth, milk, agar, Dunham's peptone solution, cotton seed meal, and egg, in which several different proteins are involved. The growth of the organism on egg increased the HCN production decidedly. This was true for all strains of *pyocyaneus* tried. This increase did not diminish when it was subsequently grown on other protein media.

Several other strains of *B. pyocyaneus* were tested for HCN production, all of which returned positive results. They were from the following sources: (1) University of Chicago stock culture; (2) egg which had been frozen two and one-half years; (3) soil; (4) Kaw river.

The stock culture of *B. pyocyaneus* from the University of Chicago, when first planted into gelatin, gave a very weak test for HCN; but after twenty-four to forty-eight hours' growth in egg, it became a strong producer of hydrocyanic acid. It would seem that the organism, living as a saprophyte, is better prepared to decompose protein substances with the production of HCN, than when living as a parasite. A rabbit was injected intraperitoneally with several cubic centimeters of a twenty-four-hour broth culture of the University of Chicago strain. After three days the rabbit died. An autopsy showed general peritonitis. Transfers made from the heart's blood gave a pure culture of *B. pyocyaneus*. This was grown in gelatin, but gave only a faint test for HCN which indicates that the organism living in an animal is reduced in its power to produce HCN. It was then grown for twenty-four hours on sterilized egg, when the test for hydrocyanic acid was one of the strongest obtained. Subsequent transfers from the egg to gelatin showed no diminution of the production of HCN. An attempt to grow the organism on Jordan's synthetic media² was unsuccessful.

B. Pyocyaneus was not the only organism found which produced HCN from proteins. Miss Myrtle Greenfield, bacteriologist for the State Water Survey, isolated an organism from soil, the name of which has not been determined. It was a strong HCN producer. The characteristics of the organism are as follows: long rod; rounded ends; no spores; very motile; takes all ordinary stains; Gram negative; abundant growth on all ordinary media; growth on agar glistening; orange pigment diffusing rapidly through agar, gelatin, potato and milk; deep colonies in agar fusiform; liquefies gelatin rapidly; gelatin stab infundibuliform. On broth and milk, a delicate pellicle is formed, and the media are colored from the surface down. Milk is gradually peptonized. Litmus is reduced. All media are made slightly alkaline. There is no diastatic action on potato starch. A slight amount of nitrite is formed. The organism grows best at 20° and is aerobic. No acid or gas is formed in dextrose, lactose, saccharose or glycerin broth.

² *Journ. of Exp. Med.*, iv, p. 629, 1899; Jordan: *Botanical Gazette*, xxvii, p. 9, 1899.

A culture of *Bacillus violaceus* from the American Museum of Natural History, was also found to produce HCN in gelatin and egg. It is very possible that other chromogens may have this same property.

A number of liquefying organisms were tested and indications of the formation of hydrocyanic acid were obtained although the tests were influenced by ammonia and sulphuretted hydrogen. They will be examined more carefully.

There has been much work done on the production of HCN from grain, beans, linseed meal, germinating *Sorghum vulgares*, and other protein-containing substances. In most cases, the production of HCN is attributed to an enzyme. Apparently all of the workers were using non-sterile material, which could have been easily contaminated by an HCN-producing organism.³

The authors are continuing the investigation with the intention of publishing more complete information concerning the decomposition of protein substances with the production of HCN due to the microorganisms mentioned in the body of the paper.

³ L. Guignard: HCN in Beans, *Recueil actes off. et doc. interessant hyg. pub., travaux Conseil Superieur Hyg. Pub. France*, 1909; S. J. M. Auld, Jr.: HCN in Linseed Cake and Other Feeding Stuff, South East Agricultural College, Wye, England, No. 20, pp. 289-320, 1911; C. Ravenna (Univ. of Bologna): HCN from Seeds of Sorghum, *Atti R. accad. dei Lincei*, xix, II, pp. 356-361; HCN from Seeds of Sorghum, *Gaz. chim. ital.*, xli, II, pp. 74-81; C. Ravenna and M. Zamorani (Lab. Agric. Chem.): Physiological Function of HCN in Plants, *Chem. Zentralbl.*, i, p. 113, 1910; M. J. Offner: HCN in Fungi, *Bull. soc. mycol. de France*, xxvii, pp. 342-5; S. J. M. Auld, Jr.: Formation of HCN in Linseed Cake and other Food Stuffs, London Board of Agriculture, No. 6, pp. 446-460; No. 8, pp. 657-660, 1912; C. D. Londer: Formation of HCN in Linseed Cake and other Food Stuffs, London Board of Agriculture, No. 11, pp. 904-907, 1911.

CONTRIBUTIONS TO THE CHEMICAL DIFFERENTIATION OF THE CENTRAL NERVOUS SYSTEM.

III. THE CHEMICAL DIFFERENTIATION OF THE BRAIN OF THE ALBINO RAT DURING GROWTH.

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(Received for publication, July 1, 1913.)

INTRODUCTORY STATEMENT.¹

The transformations which occur in the brain during growth offer a particularly enticing field for the study of chemical differentiation, not alone because of the very great interest attaching to the solution of the problem of the chemical basis of its functions, but because its structural differentiation during growth is very marked. On the one hand there is the formation of a large amount of new material composing the medullary sheath of the nerve fibers, and, on the other hand, the appearance of a quantity of a peculiar supporting tissue, the neuroglia. The chemical changes during growth should, therefore, be very marked; and it is of interest to discover how far our chemical methods enable us to follow such obvious structural modifications.

¹Waldemar Koch died at Chicago February 1, 1912. As Associate in Biological Chemistry at the Wistar Institute of Anatomy and Biology, he spent the autumn of 1910 and of 1911 in Philadelphia working mainly on matters connected with this research. This paper has been prepared in considerable part from results of analyses made by me under the direction of my brother, and from a manuscript written by him. Many additional analyses, which he had planned, have been made and incorporated into the series. The interpretations of the results have been left to a large extent, in his words. I have been assisted in its preparation for publication by Professor A. P. Mathews and Dr. H. H. Donaldson, the aid of both of whom I gratefully acknowledge.—M. L. KOCH.

The selection of chemical methods for such a study was largely guided by the principle now coming to be generally accepted, namely, that in living matter we are not dealing with an aggregation of more or less similar, highly organized and necessarily complex molecules (Riesenmolekül of Pflüger), but rather, with a more or less heterogeneous substratum in which dissimilar and not necessarily highly complex molecules, or their dissociated particles, are engaged in a series of correlated chemical reactions. The larger aggregates may be conceived as either not taking part directly in chemical activity, or as helping in the control and localization of the chemical reactions, just as in a photographic dry plate, the presence of the gelatin makes possible a high degree of localization of the photo-chemical reaction. We aimed, therefore, to stop the chemical activities at definite given stages during the growth period and then to observe the differences which could be demonstrated. From such data we then drew conclusions as to the nature of the transformations which had occurred in the interval.

The methods of collecting the material were devised with this end in view, namely, to stop all chemical activity as rapidly and completely as possible. The sources of error due to post mortem changes then became constant, and we are in reality following a principle that has long been in use in histological studies. For the preserving agent, alcohol was selected, as it is the least apt to interfere with the further chemical procedure, and, in fact, treatment with alcohol represents a step in the process.

In the selection of the chemical methods for this series two points were kept in mind:

1. The necessity of correlating the chemical observations with the known facts of structure, to the interpretation of which they should add a greater precision. As an example of this, there were studied the sulphatides (lipoid sulphur) which are intimately associated in the nervous system with the sheaths of the medullated nerve fibers.

2. The collection of data, which, correlated with function, would give the physiologist a better knowledge of the nature of his material and thus enable him to do more than speculate as to the probable nature of the processes involved in the phenomena

he is observing. As an example of this, there was studied the ratio between neutral sulphur and protein sulphur, a ratio which correlates closely with the decrease in metabolic activity associated with the growth of the nervous system from birth to maturity.

The general plan of the chemical technique has been first to block out the material into larger groups of substances and then carry the procedure of separation into greater detail. The necessity of working with data which represent something definite from the point of view of the chemist, has also been kept in mind.

The following outline illustrates the extent to which the chemical procedure has been carried up to the present.

Outline illustrating the separation of constituents by the method employed,² classified according to their state of aggregation.

	ENCEPHALON DIVIDED BY STATE OF AGGREGATION INTO:			
	COLLOIDAL (FRACTION 1 AND 4)		NON-COLLOIDAL (FRACTION 2 AND 3)	
	Proteins (Fract. 4)	Lipoids (Fract. 1)	Organic Extractives (Fract. 2 and 3)	Inorganic Constituents (Fract. 2 and 3)
Proximate constituents	(Include supporting structures)	Phosphatides Cerebrosides Sulphatides {Cholesterol Undetermined		Sodium Potassium Calcium Magnesium Chlorides
Sulphur combinations	Protein S	Lipoid S	Neutral S	Inorganic S (sulphates)
Phosphorus combinations	Protein P	Lipoid P	Organic extractives P	Inorganic P (phosphates)

For an explanation of the chemical procedure followed for this separation the following outline has been inserted.

² The method employed for this separation is described in an earlier paper by W. Koch and coworkers: *Journ. of the Amer. Chem. Soc.*, xxxi, pp. 1342-1361, 1909.

*Moist Brain Tissue: Add alcohol and extract alternately with alcohol and ether.*³

EXTRACT (FRACTION 1 AND 2)		RESIDUE (FRACTION 3 AND 4)	
EVAPORATE TO DRYNESS, EMULSIFY WITH WATER, PPT. WITH CHCl_3 IN 0.5 PER CENT HCl SOLUTION		DRY, WEIGH AND EXTRACT WITH HOT WATER	
Ppt. (Fract. 1):	Filtrate (Fract. 2):	Filtrate (Fract. 3):	Residue (Fract. 4):
Lipoids	Organic extractives Inorganic constituents	Organic extractives Inorganic constituents	Proteins

Organic extractives in Fraction 2 and 3 are equal to total organic extractives.

Inorganic constituents in Fraction 2 and 3 are equal to total inorganic constituents.

Fraction 1 and 2 are soluble in alcohol (85-95 per cent).

Fraction 3 is insoluble in alcohol; soluble in hot water.

Fraction 4 is insoluble in alcohol and hot water.

For a clearer understanding of the terms used in this series of papers, the following interpretation of the *chemical nature, anatomical distribution, and physiological significance* of the substances determined, with special reference to the nervous system based both on the studies already made and those presented in this paper, is given below.

Proteins.

Chemistry. These represent complex combinations of amino-acids rendered insoluble in water by coagulation with hot alcohol. This fraction has been exhaustively extracted with hot alcohol and should retain only traces of lipoids and fats. The nucleoproteins and the neurokeratin are included in this fraction.

Anatomical distribution. In the part of the nervous system rich in cells (cortex) the proportion of the proteins is larger than in the white matter. Some of the nucleoproteins are supposed to be associated with the chromatin and Nissl substance of the nerve cell. The remainder of the nucleoproteins are represented by the nuclei of the glia cells scattered through-

³ Although ether is used in the extraction following the first alcohol, it does not remove any considerable amount of material and need not be considered in the above scheme.

out the nervous system. Neurokeratin occurs in the medullated sheath of the nerve fiber. The other proteins occur in the axon of the nerve fiber as well as in the cell body and its dendrites.

Physiological significance. The proteins have usually been considered as the essentially living part of the protoplasm, but some of them, like neurokeratin, are undoubtedly inactive and represent supporting structures. The same may be said of the proteins which make up the fibers of the glia cells. It is therefore impossible to tell at the present time to just what extent and in what proportion the proteins are involved in the chemical activities of the nervous system. The significance of the neutral sulphur compounds, which represent simpler cleavage products of the larger protein aggregates, will be discussed later as having an important bearing on this point (see p. 431).

Phosphatides.

Chemistry. These represent complex combinations of fatty acids, phosphoric acid, glycerin, and nitrogen complexes of the nature of choline, and include among other things lecithin and kephalin. The chemistry of this group is very much in need of revision, as some of its members are not so simple as the older work of Hoppe-Seyler has led us to infer. The group does not include lecithin in combination with sulphur or cerebrin. The phosphatides as here given are calculated from the phosphorus of the lipid fraction on the assumption that they have an average molecular weight of 800. Correction must be made for the phosphorus of the sulphatides.*

Anatomical distribution. Comparison of cortex and corpus callosum⁵ indicates that the phosphatides are not very differently distributed between cell body and nerve fiber. Analyses of the brain at a period when medullation has not begun, but when the cell processes are growing freely, indicate that the phosphatides are largely associated with the axon. If mitochondria consist largely of phosphatides, as has been suggested, the observations of Cowdry would give us a picture of their distribution in the cell body. The absence of mitochondria in the axon, which is known to contain phosphatides, would not argue against this, as there is some evidence that the phosphatides of the processes and the cell body are different in their behavior.

Physiological significance. The phosphatides, like the proteins, may be considered to be intimately associated with the vital processes of the living protoplasm. Their colloidal nature and relation to inorganic ions, as well

* *Calculations for phosphatides.* The total lipid phosphorus found times 25.77 gives the phosphatides, on the basis that 3.88 per cent of the phosphatides consist of phosphorus. Since 51.2 per cent of the sulphatides are phosphatides, that amount was deducted from the total phosphatides found. The difference was considered as free phosphatides.

⁵ Koch, W.: *Amer. Journ. of Physiol.*, xi, pp. 326-328, 1904.

as their instability towards heat,⁶ lend support to this idea. They probably occur largely in the cytoplasm, cell body and its branches, where they may act as oxygen carriers, as has been suggested by the work of Koch and Mostrom.⁷

Cerebrosides.

Chemistry. Complex combinations of fatty acids, galactose, and possibly other hexoses with a nitrogen complex of the nature of sphingosine. The cerebrosides are calculated from the lipid sugar on the assumption that they yield on hydrolysis 21.8 per cent of reducing sugar, the amount found by Thierfelder in his cerebron. Correction must be made for the cerebrin content of the sulphatides.⁸

Anatomical distribution. Although the cerebrosides are occasionally met with in other tissues, they occur in largest amount in the medullated nerve fiber, and their quantity increases as medullation proceeds. The rather large amount found in the cortex⁹ on chemical analysis indicates that they may predominate in the fibers of that region.

Physiological significance. As laid down in the medullated nerve fiber, the cerebrosides most probably serve only a mechanical function and are not available as sources of energy in spite of their carbohydrate and fatty acid content.

Sulphatides.

Chemistry. These represent the combination of a phosphatide with a cerebroside by means of a sulphuric acid group in ester combination.¹⁰ The sulphatides are estimated from the lipid sulphur on the basis of a sulphur content of 2 per cent, based on the analysis of a purified compound.¹¹

⁶ Koeh, W and Koeh, M. L.: this *Journal*, xiv, pp. 281-282, 1913.

⁷ Koeh, W and Mostrom, H. T.: *Journ. of Pharm. and Exp. Ther.*, ii, No. 3, p. 265, 1910.

⁸ *Calculations for cerebrosides.* The cerebrosides, from the lipid fraction, on hydrolysis for twenty-four hours with a weak solution of HCl (75 cc. of water containing 3 cc. concentrated HCl), yield 21.8 per cent by weight galactose. The calculations for cerebrosides were made on the assumption that galactose and glucose were equivalent in reducing power and the weight of galactose was thus determined from Munson and Walker's tables for glucose. (*Journ. Amer. Chem. Soc.*, xxviii, p. 663). The corrected weight of total galactose to cerebrosides was then made on the basis that 21.8 per cent of the latter is galactose. Finally since 42.9 per cent of the sulphatides consist of cerebrosides this amount was deducted from the total cerebrosides found. The difference was considered as cerebrosides.

⁹ Koch, W. and Mann, S. A.: *Archives of Neurology and Psychiatry*, iv, p. 33, 1909.

¹⁰ Koch, W.: *Zeitschr. f. physiol. Chem.*, lxx, p. 94, 1910.

¹¹ *Calculations for sulphatides.* These are considered to be of the general formula:

If it were desirable to recognize the chemical identity of the much abused protagon, the sulphatides might be considered as purified products. Protagon could be much more safely calculated from the lipoid sulphur than from the lipoid sugar as Noll¹² has attempted. The sulphur content of protagon preparations, when it has not been simply ignored, is variously reported as 0.5 and 1.0 per cent.

Anatomical distribution. The sulphatides, like the cerebrosides, increase parallel with the growth of the medullary sheath and may be considered as essential constituents of that structure. The fact that the sulphatides, as the result of more recent work, have been found to be pretty generally distributed in other tissues, indicates that they might occur in the cell body of the neurone, although a comparison of the analyses of cortex and corpus callosum does not make this very probable. The sulphatides have an important function in the maturing of the nerve fiber and give the Weigert staining reaction in a very characteristic manner.

Physiological significance. Their colloidal nature and the peculiar combination into which the sulphatides enter with potassium, suggests that they may have an important relation to the nerve impulse and to the phenomena of conductivity in general.

Organic extractives and inorganic constituents.

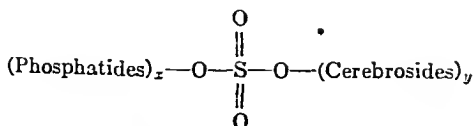
Chemistry. This group represents essentially the water-soluble, non-colloidal constituents of the nervous system. The older method of estimating the inorganic constituents by the ash has been abandoned as too inaccurate. The principal reason for reporting the above group is to give an idea of the ratio between the colloidal and non-colloidal constituents.

Anatomical distribution. The group occurs in large quantity in the cell body, although some is also present in the axon of the nerve fiber.

Physiological significance. This group is a rough index of the amount of metabolic activity going on in the tissue, as it represents at the same time the end products of chemical activity, as well as the culture media from which the more complex combinations are built up.

Undetermined (cholesterol).

This fraction is represented in the nervous system to a certain extent by cholesterol, which has not been directly estimated. Besides this, how-



containing 2.0 per cent sulphur, 42.9 per cent cerebrosides, and 51.2 per cent phosphatides. Then we have,

$$\frac{(\text{Lipoid sulphur} \times 50)}{\text{weight of dry substance}} = \text{per cent of sulphatides in dry substance.}$$

¹² Noll, A.: *Zeitschr. f. physiol. Chem.*, xxvii, p. 370, 1899.

ever, all the errors of analysis, as well as of such calculations as are based on assumed factors, enter into this fraction. After accounting for the cholesterol in the brain of the 50 and 100 mm. pig fetus,¹³ in which this was estimated directly by Mendel, there remained undetermined 2 to 3 per cent of the total solids. Considering the number of groups estimated, this is not a very discouraging result. (In other tissues, which contain little cholesterol, the undetermined is recorded as neutral fat.)

Anatomical distribution. Cholesterol is principally of interest as a constituent of the medullary sheath to which it adds a sort of mechanical stability. But it is present in the cell bodies also, possibly contributing to the cell membranes. According to Lorrain Smith¹⁴ it is one of the substances responsible for the color which the medullary sheath gives with Weigert's stain.

Total sulphur and total phosphorus.

It may not be out of place to state briefly the reasons for selecting these two elements for special determination in preference to others. As far as the phosphorus is concerned, the importance of the nucleins to all living cells and the phosphatides to the nervous system in particular, amply justify its selection. The reason for selecting sulphur in preference to the much more generally studied nitrogen, may, however need a word of explanation.

Nitrogen is studied for two reasons: because it is an important element in the building up of the proteins, and because it is easy of estimation.

Sulphur is just as characteristic of proteins, in fact more so, as it does not enter into the non-protein groups such as the nucleic acids. Among the lipoids, too, sulphur enters into only one group, the sulphatides, while nitrogen occurs in all except cholesterol.

In other words, to estimate sulphur in the protein fraction is to estimate an element essentially characteristic of the more truly protein part. To estimate it in the lipid fraction, enables one to distinguish one particular, and, as growth curves show, a very interesting group of lipoids. Besides, as has already been pointed out in a previous paper¹⁵ sulphur occurs in the tissues in several states of oxidation and thus gives us some indication of the intensity of reactions of oxidation which are so important to growing tissues, and about which we know so little. It seems wise therefore to estimate the sulphur, and in case there are any special reasons to study nitrogen, to study it rather in the form of one of its definite groups of compounds such as the purine bases or the amino-acids.

¹³ Koch, Mathilde L.: this *Journal*, xiv, pp. 267-279, 1913.

¹⁴ Smith, Lorrain: *Journ. of Path. and Bact.*, xv, pp. 179-181, 1911.

¹⁵ Koch, W. and Upson, F. W.: *Proc. Soc. for Exp. Biol. and Med.*, vii, pp. 5-6, 1909.

Distribution of sulphur.

Chemistry. **PROTEIN S.** This group represents sulphur in various amino-acid combinations such as cystine or cysteine. The proteins in which this sulphur fraction is found have been coagulated and rendered insoluble in water by treatment with hot alcohol.

LIPID S. Ethereal sulphuric acid combinations are discussed under sulphatides.

NEUTRAL S. This group of compounds represents the total non-colloidal, water-soluble combinations of sulphur, minus the inorganic sulphates. As far as studied, they resemble in all their reactions a similar group found in the urine and called by Bondzynski proteinic acids. They represent, probably, larger cleavage products of the protein molecule or complex non-coagulable, water-soluble polypeptides somewhat altered by processes of oxidation. The sulphur of this fraction is represented essentially by compounds included among the organic extractives, and the sulphur is most often in an oxidized form like taurine or ethereal sulphate.

INORGANIC S (inorganic sulphates). Derivatives of sulphur directly precipitated by barium chloride in hydrochloric acid solution.

Anatomical distribution and physiological significance. The **LIPID S**, as has already been mentioned under the sulphatides, represents an essential constituent of the medullary sheath. The proportion in which it occurs in the sheath can be considered as a measure of the maturity of the sheathing substance.

The **PROTEIN S** AND **NEUTRAL S** will be considered together as they bear an important relation to one another and as the combinations in which they occur are essential constituents of all living cells. As has already been stated, the study of these two groups of sulphur compounds gives us a means of investigating the protein metabolism of the central nervous system during its growth period.

TABLE I.

A comparison of neutral sulphur with protein sulphur in the brain of the albino rat at different ages (figures in per cent of total sulphur).

	PROTEIN SULPHUR	NEUTRAL SULPHUR
1 day.....	30.5	48.2
10 days.....	44.2	45.4
20 days.....	56.4	28.6
40 days.....	63.7	18.2
120 days.....	61.8	18.7
210 days.....	63.8	14.5

During the early stages when growth is proceeding rapidly and chemical activities may be considered to be at their height, the proportion of

non-colloidal, relatively smaller, neutral sulphur molecules is at a maximum. This is what we should expect when we consider living matter not as a collection of highly organized molecules, but rather as a heterogeneous substratum in which relatively smaller molecules or their dissociated products are engaged in chemical transformations. As the tissues grow and become more highly differentiated and mature, more and more protein is laid down as structural material, and the proportion is shifted in the direction of the protein sulphur. A comparison of the cortex of the human at two years and at maturity illustrates this point.¹⁶

2 years' cortex.....	Protein S, 63; Neutral S, 22.
19 years' cortex.....	Protein S, 73; Neutral S, 12.

The change suggests, therefore, a decrease in chemically active material associated with the increasing complexity of the tissue. Such data as we have at hand indicate that we have in the protein sulphur and neutral sulphur ratio a valuable means of measuring the relative growth intensity of the nervous system at different periods during its development after the state of cell division has practically ceased.

There might be another way of measuring this intensity of chemical activity, namely, by means of the inorganic sulphates, which represent the end products and the final state of oxidation of the compounds involved in these reactions, but they are eliminated rather easily from the cell, and it is therefore difficult to attach any significance to their variations.

Distribution of phosphorus.

Chemistry. **PROTEIN P.** This group represents phosphorus largely in combination as nucleic acid. In the nervous system this nucleic acid is combined with such a very large amount of protein¹⁷ that the per cent of phosphorus in the resulting nucleoprotein drops to 0.57 per cent as compared with 3 to 4 per cent in such a tissue as the pancreas.

LIPID P. Already discussed under phosphatides.

WATER-SOLUBLE P. This group includes non-colloidal, water-soluble organic combinations of phosphoric acid and inorganic phosphates. On account of the relative ease with which the organic extractive combinations of this form of phosphoric acid break down, it is difficult to estimate the proportion which is in organic combination. The results which are so far recorded represent, therefore, rather the possible maximum, than a very close approach to the actual value. (See articles of Grindley,¹⁸ Trowbridge,¹⁹ and Forbes.²⁰)

¹⁶ Koch, W. and Mann, S. A.: *Journ. of Physiol.*, xxxvi, p. 2, 1907.

¹⁷ Also accounts for poor staining reaction of neurone nucleus.

¹⁸ Grindley: *Journ. of Amer. Chem. Soc.*, xxviii, pp. 25-63, 1906.

¹⁹ Trowbridge, P. F. and Francis, C. K.: *This Journal*, vii, pp. 481-501, 1910.

²⁰ Forbes, E. B.: *Ohio Agric. Exp. Sta. Bulletin* 215, 1910, pp. 459-489.

Anatomical distribution and physiological significance. The protein phosphorus is largely associated with the nucleic acid of the nucleus. In the nervous system it is also supposed to be associated with the Nissl substance, but this is still a doubtful matter. The accuracy with which we can estimate nuclear material in the anatomical sense from such a figure as the protein phosphorus is difficult to determine, as there are three complicating factors.

1. The possibility that the nucleus, as an anatomical unit, contains other compounds besides nucleoproteins.

2. The fact that nucleic acid itself may be associated with very widely varying quantities of protein.

3. The possibility that substances yielding a protein phosphorus fraction may occur in the cytoplasm.

Observations by Miescher²¹ on the sperm, however, very strongly suggest that protein phosphorus is largely associated with the nucleus, while lipid phosphorus is largely associated with the cytoplasm.

As regards the *water-soluble phosphorus*, the principal point of interest, just as in the case of the neutral sulphur, is its ratio to the protein or colloidal forms of phosphorus. Thus in a study on a lower plant form (*Aspergillus niger*) Koch and Reed²² could demonstrate that under extreme conditions, such as can only be realized with plant material, it is possible to carry the growth processes to such a point that all the non-colloidal, water-soluble phosphorus is converted into colloidal combinations. At such a point the growth of the plant comes to a stop.

The function of the *inorganic phosphates* in maintaining the neutrality of protoplasm as suggested by Henderson²³ is also a point of interest, although of less importance to the nervous system than to muscle tissue.

Inorganic constituents.

Chemistry. The inorganic constituents found in the nervous system are the cations Na, K, Ca, Mg, Fe, and the anions Cl, SO₄, PO₄.

In the method devised for this study the usual method of estimating these constituents by the ash was abandoned on account of the fact that by the process of ashing, the relation of cations to anions is profoundly altered. As a result of this precaution, the interesting fact has been clearly demonstrated that a large proportion of the cations, especially sodium and potassium, occur combined with complex anions, sometimes colloidal in nature.

The work of Pike²⁴ has brought to light the interesting point that in the nervous system the sodium and potassium, more especially the latter,

²¹ Miescher, F.: *Hoppe-Seyler's Med.-chem. Unters.*, p. 452.

²² Koch, W. and Reed, H. S.: *this Journal*, iii, p. 49, 1907.

²³ Henderson, L. J.: *this Journal*, vii, pp. 29-35, 1910.

²⁴ Koch, W. and Pike, F. H.: *Journ. of Pharm. and Exp. Ther.*, ii, pp. 245-248, 1910.

are combined with such lipoids as the sulphatides and kephalines (a subgroup of phosphatides), while the Ca and Mg have more tendency to remain combined with the proteins.

Anatomical distribution and physiological significance. Very little is known of the anatomical distribution of the salts except as shown by the work of Macallum²⁵ which demonstrates that chlorides and potassium are associated with the nerve fiber. According to Alcock,²⁶ potassium is supposed to play an important rôle in the propagation of the nerve impulse.

With this introductory statement of the anatomical distribution and physiological significance of the substances quantitatively determined, we may now present the results of a study of their variation during the growth of the brain.

The brain of the albino rat was selected for this study, for the reasons already presented in the first paper of this series.²⁷

From a comparison of the brain of the albino rat at birth and the brain of the fetal pig, it was found that the brain of the new born rat is as young nervous material as can conveniently be analyzed at present. It forms therefore a suitable starting point for this study of chemical differentiation during growth. The analyses reported in this paper are those of the brains of rats aged respectively, 1, 10, 20, 40, 120, and 210 days. The results show that it was possible to follow closely the various structural changes which occur during the differentiation of the growing nervous system

The material was furnished by the Wistar Institute of Anatomy; the brains being collected and analyzed in the manner already detailed.²⁸ Koch's quantitative methods were used.²⁹

RESULTS OF ANALYSES.

The results of analyses are embodied in Table II. Duplicate analyses have been carried on throughout, and are summarized in Table III. This table gives the averages of the analyses, except

²⁵ Macallum, A. B.: *Journ. of Physiol.*, xxxii, pp. 95-128, 1905; Macallum, A. B. and Menten, M. L.: *Report 75th Meeting British Assoc. Adv. Sci.*, p. 555, 1906.

²⁶ Alcock, N. H.: *Journ. of Physiol.*, xxxix, pp. 402-410, 1911.

²⁷ Koch, Mathilde L.: *this Journal*, xiv, pp. 267-279, 1913.

²⁸ Koch, Mathilde L.: *loc. cit.*

²⁹ Koch, W.: *Journ. of Amer. Chem. Soc.*, xxxi, pp. 1335-1364, 1909.

in two instances where the value from one analysis only is preferred. Table IV gives the absolute weights of these constituents, as found in one brain; while Table V gives the ratio of increase of the different constituents, taking the amount of each constituent in the brain of the rat at birth to be unity and determining the number of times each constituent had increased at successive ages from birth to maturity. For comparison there is given in Table II one analysis of the spinal cord at 120 days.

DISCUSSION OF RESULTS.

The growth of the nervous system from the first laying down of the neural canal to maturity may be divided into four periods. The first period, during which cell division is the most characteristic feature, lasts to about birth. A short time before birth cell division begins to decrease. The chemical changes during this first period were not studied directly in the albino rat for the reasons stated in the first paper³⁰ but the composition of the nervous system in this primitive, undifferentiated state may be seen in the analysis of the fetal pig brain reported in the first paper of the series. At this time phosphatides are present, sulphatides are relatively less important and cerebrosides are entirely lacking; proteins, phosphatides, extractives, salts and water are the predominant constituents of the tissue.

The second period (see Table VI) lasts from birth for about ten days, when the third period begins. The second period is characterized structurally by the development of fibers from the cells and the increase in their size. Donaldson has estimated that the number of nerve cells does not increase more than 3 to 6 per cent during this period, but the cells do add to the number and size of their branching processes. This period, as may be seen from Table VI, is one of intense growth of all the solid constituents. The proteins continue throughout this period to be formed at a very rapid rate, 4-5 mgms. being laid down per day. Cerebrosides are either absent entirely, or present in very small quantities.

In the third period, that of most rapid growth, from the tenth to the twentieth day, medullation begins. There is a wonderful

³⁰ Koch, M. L.: this *Journal*, xiv, p. 279, 1913.

TABLE II. Continued.

Distribution of sulphur in per cent of total S.

Protein S.....	31.1	30.0	48.6	44.2	57.5	55.3	65.1	62.4	61.2	62.4	63.8	53.7
Lipoid S.....	3.2	2.8	2.2	6.1	6.7	7.5	9.2	10.1	12.8	12.5	15.6	30.9
Neutral S.....	49.1	47.3	45.1	45.4	29.7	27.5	17.0	19.3	19.2	18.3	14.5	10.3
Inorganic S.....	16.6	19.9	4.1	4.3	6.1	9.7	8.7	8.2	6.8	6.8	6.1	5.1

Distribution of phosphorus in per cent of total P.

Protein P.....	13.3		13.0	13.9	6.0	5.8	9.9	7.5	7.4	7.3	6.8	5.6
Lipoid P.....	33.2	33.0	33.8	36.1	52.2	53.5	56.1	58.5	65.8	62.3	67.6	77.4
Water Sol. P.....	53.5	53.6	53.2	50.0	41.8	40.7	34.0	34.0	26.8	30.4	25.6	17.0

* Cerebromides not determined in brains at birth and 10 days. Probably none present at this age.

† By difference.

‡ Indicates doubtful result.

§ Taken from W. 8.

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TABLE III.

The relative proportions of the constituents of the brain of the albino rat at different ages (averages from Table II).

	AGE IN DAYS					
	1	10	20	40	120	210
Moist weight of one brain in grams..	0.25*	0.86†	1.28*	1.38*	1.60*	1.67†
Solids in per cent.....	10.42	12.5	17.5	20.34	21.65	21.9
Dry weight of one brain in grams....	0.026	0.107	0.224	0.281	0.347	0.365
Number of brains in each sample....	100	40	54	35	30	31
Laboratory Number.....	W. 16, 24	W. 40	W. 17, 25	W. 28, 29	W. 7, 8	W. 13

*Constituents in per cent of total solids.**

Proteins.....	58.25*	56.5†	53.3*	48.4*	47.6*	48.5†
Phosphatides.....	15.2	12.3	21.4	21.8	21.6	22.0
Cerebrosides.....			3.0	5.9	8.4†	8.4‡
Sulphatides.....	1.45	2.6	2.5	2.55	3.55	4.5
Organic extractives.....	17.9	15.1	14.55	14.85	9.75	9.8‡
Inorganic constituents.....						
Cholesterol (undetermined)§.....	7.2	13.5	5.25	6.5	9.1	6.8
Total sulphur.....	1.00	0.83	0.70	0.55	0.56	0.58
Total phosphorus.....	1.87	1.48	1.66	1.52	1.42	1.39

Distribution of sulphur in per cent of total S.

Protein S.....	30.5	44.2	56.4	63.75	61.8	63.8
Lipoid S.....	3.0	6.1	7.1	9.65	12.7	15.6
Neutral S.....	48.2	45.4	28.6	18.15	18.7	14.5
Inorganic S.....	18.3	4.3	7.9	8.45	6.8	6.1

Distribution of phosphorus in per cent of total P.

Protein P.....	13.3	13.45*	5.9	8.7	7.3	6.8
Lipoid P.....	33.2	34.95	52.85	57.3	64.1	67.6
Water sol. P.....	53.5	51.6	41.25	34.0	28.6	25.6

* Record from average duplicate analyses.

† Record from one analysis only.

‡ Taken from analysis, W. 8.

§ Obtained by difference.

TABLE IV.

Absolute weights, in milligrams, of the constituents of a single brain of the albino rat at different ages (prepared from Table III).

	AGE IN DAYS					
	1	10	20	40	120	210
Moist weight of one brain in grams....	0.25	0.86	1.28	1.38	1.60	1.67
Solids in per cent....	10.42	12.5	17.5	20.34	21.65	21.9
Dry weight of one brain in grams....	0.026	0.107	0.224	0.281	0.347	0.365
Laboratory Number....	W. 16, 24	W. 40	W. 17, 25	W. 23, 29	W. 7, 8	W. 13

Absolute weights in milligrams.

Proteins (1)†.....	15.14*	60.45†	119.4*	136.0*	165.2*	177.0†
Phosphatides (2)...	3.95	13.16	47.9	61.3	74.95	80.3
Cerebrosides (3)....			6.7	16.6	29.15	30.66
Sulphatides (4)....	0.38	2.78	5.6	7.2	12.3	16.4
Organic extrac- tives.....	4.65	16.16	32.6	41.7	33.8	35.8
Inorganic constit- uents.....						
Cholesterol unde- termined (5)....	1.87	(14.45)	11.7	18.2	31.6	24.8
Total sulphur.....	0.26	0.90	1.57	1.54	1.94	2.12
Total phosphorus...	0.48	1.6	3.72	4.30	4.93	5.07

In absolute weight in milligrams of sulphur.

Protein S (1S)§....	0.079	0.398	0.885	0.982	1.199	1.352
Lipoid S (4).....	0.008	0.054	0.111	0.149	0.246	0.330
Neutral S (6).....	0.125	0.409	0.449	0.279	0.363	0.307
Inorganic S (7)....	0.047	0.039	0.122	0.130	0.132	0.129

In absolute weight in milligrams of phosphorus.

Protein P (1P).....	0.064	0.215*	0.220	0.374	0.360	0.345
Lipoid P (2).....	0.161	0.558	1.964	2.464	3.160	3.427
Water sol. P (8)....	0.260	0.826	1.532	1.462	1.410	1.298

* Record from average duplicate analyses.

† Record from one analysis.

‡ Figures in parentheses in this section refer to Chart III.

§ Figures in parentheses in this and the following sections refer to Chart IV.

TABLE V.

The ratio of the increase of the constituents of the brain of the albino rat at different ages, taking the amount of each constituent found in the brain at birth as unity (prepared from Table IV).

	AGE IN DAYS					
	1	10	20	40	120	210
Total Solids.....	1	4.0	8.6	10.8	13.3	14.0
Proteins.....	1	4.0	7.9	9.0	11.0	11.7
Phosphatides.....	1	3.3	12.0	15.5	19.0	20.3
Cerebrosides.....						
Sulphatides.....	1	7.4	14.8	19.0	32.6	43.5
Organic extractives.....	1	3.5	7.0	8.9	7.2	7.7
Inorganic constituents.....						
Cholesterol (undetermined).....	1	7.7	6.2	9.0	16.9	13.2
Total sulphur.....	1	3.4	6.0	5.9	7.4	8.0
Total phosphorus.....	1	3.2	7.6	8.8	10.1	10.4
Protein S.....	1	5.0	11.1	12.3	15.0	17.0
Lipoid S.....	1	6.9	14.3	19.1	31.5	42.3
Neutral S.....	1	3.3	3.6	2.2	2.9	2.5
Inorganic S.....	1	(1.3)	2.6	2.7	2.8	2.7
Protein P.....	1	4.1	4.3	7.2	7.0	6.7
Lipoid P.....	1	14.3	15.3	19.2	24.7	26.8
Water sol. P.....	1	3.9	7.4	6.9	6.8	6.2

TABLE VI.

Rate of growth (milligrams formed per day) of different constituents in a single brain of the albino rat at different age periods (prepared from Table IV).

	AGE PERIODS					
	2	3	4			
	1-10 days	10-20 days	20-40 days	40-120 days	120-210 days	
Between.....	{					
Proteins.....		4.53	5.9	0.84	0.36	0.13
Phosphatides.....		0.92	3.5	0.67	0.17	0.06
Cerebrosides.....				0.49	0.15	0.006
Sulphatides.....		0.24	0.29	0.08	0.06	0.045
Organic extractives.....	}	1.51	1.64	0.46	0.00	0.000
Inorganic constituents.....						
Cholesterol (undetermined).....		(0.49)	(0.49)	0.32	0.17	0.000

outburst of activity in forming phosphatides which reach a maximum rate of formation of 3.5 mgms. per day. This change is no doubt correlated with the great growth of the fibers and the beginning of medullation. The organic extractives and inorganic constituents continue to be formed at the same rate since the cell bodies are increasing in size; probably not more than from 10 to 20 per cent having reached anything approaching adult size up to this time.

The sulphatides, although present in less quantity than the phosphatides, reach also their maximum rate of formation. The whole chemical picture is that of a rapid growth of protoplasm, with a change in its character owing to the increase of phosphatides. During this period the neurones increase rapidly in size.

Attention is particularly directed to the temporary great increase in neutral sulphur during these two periods of intense growth (Table IV). The significance of this has already been discussed on p. 431 *et seq.*

The fourth growth period is the period of continued medullation. This period is characterized chemically by a great reduction in the rate of formation of all substances except the cerebro-sides. These latter between 20-40 days come into view, almost equalling the phosphatides and being more than half the amount of the proteins formed at the same time. The cerebro-sides contribute a large share toward medullation. The rate of formation of the various constituents per day falls in the 20-40 day period, as compared with the 10-20 day period, in the case of the proteins to one-seventh; the phosphatides to one-fifth; the sulphatides to one-third; and the organic and inorganic extractives to one-third. The formation of the proteins decreases the most; the cerebro-sides, the least. If the rate of formation in the 40-120 day period is compared to that of the 10-20 day period it is seen that the protein formation has decreased to one-sixteenth; the phosphatides to one-twentieth; the sulphatides to one-fourth but are still increasing. On the other hand, the organic extractives and inorganic constituents have not increased at all, indicating that metabolism is much reduced in its rate and the growth of the protoplasm is much slower. During this fourth period then, the sulphatides continue to be formed at a more rapid rate, relative to their total amount, than any other constituents; and in the 120-210

day period, the total sulphatides formed surpass the cerebroside, nearly equal the phosphatides, and are more than one-third the proteins. The constant production of sulphatides is, therefore, a marked feature of late medullation, just as that of the phosphatides is of the early medullation. The sulphatides diminish in their rate of formation far less than any other constituents.

Finally we have the period from 210 days on: the period of stationary or adult life. We have no definite chemical data as to any changes occurring during this period, but from such data at hand, as the periods just studied, we can assume that the growth processes during adult life are practically stationary except perhaps a very gradual increase in the per cent of solids.

The enlargement of the brain may, therefore, in great part be accounted for chemically by the formation of the medullary sheath. Donaldson³¹ has found that some 88 per cent of the volume of the adult brain is composed of the axons and their sheaths, while the cell bodies with their dendrites and the supporting tissues together only make up the remaining 12 per cent. The axones, therefore, medullated or non-medullated, are mainly responsible for the increase of the size of the brain and for the changes which it undergoes during post natal growth. To bring more vividly before the eye the relative rate of growth of the various constituents, we have prepared Charts 1 and 2 from Tables III and IV. These charts are self explanatory. We have also prepared Charts 3 and 4, an explanation of which is given below.

Chart 3 shows the relation of lipid³² to protein. In this the weights of the several constituents are represented for one brain at each age. This chart shows that while both the proteins and the lipoids are increasing in absolute weight, the proportion of lipid to protein is becoming greater and greater as the tissue grows older. This indicates that the rate of increase for the lipoids is greater than for the proteins (also brought out in Table VI). At 120 and at 210 days we find that the lipoids and pro-

³¹ Donaldson, H. H.: *Journ. of Nervous and Mental Disease*, xxxviii, p. 260, 1911.

³² Lipoids here include the phosphatides, cerebroside, and sulphatides; cholesterol, which is classed as lipid, is here recorded in the "undetermined."

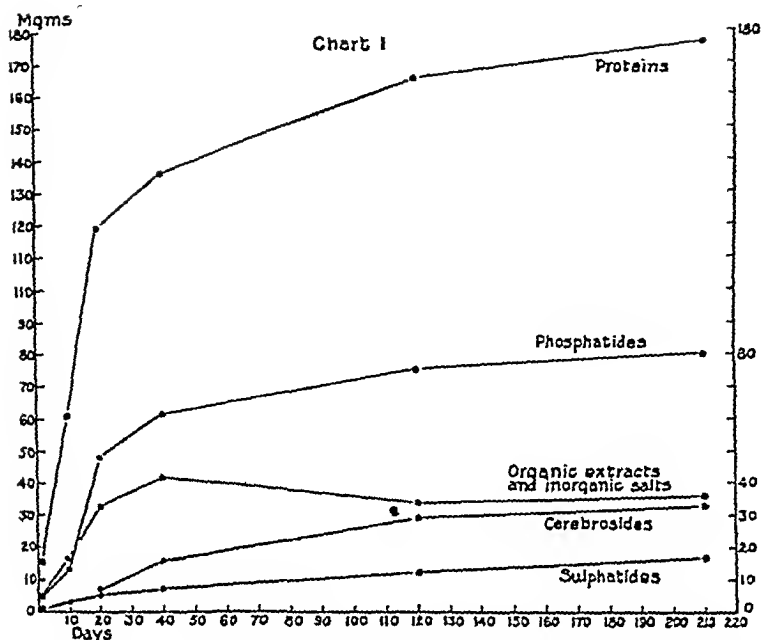


CHART 1. Shows the absolute weight in milligrams, of the constituents of a single brain of the albino rat at different ages. (Age in days along the abscissa; mgms. on the ordinate.)

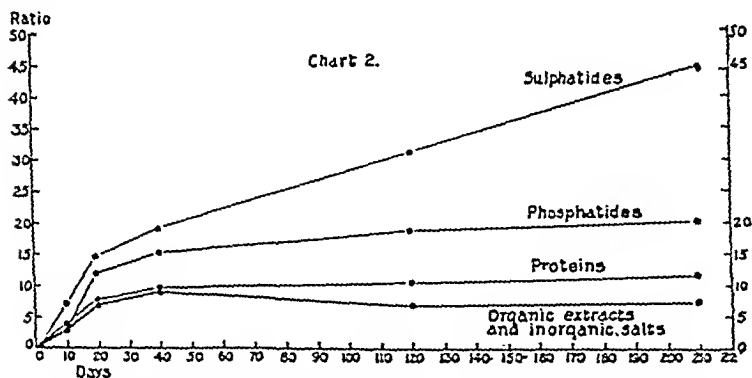
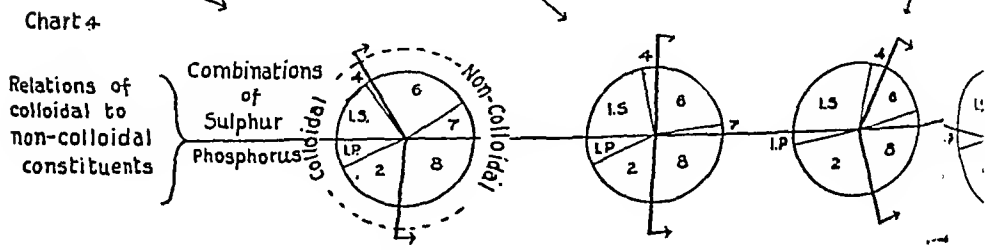
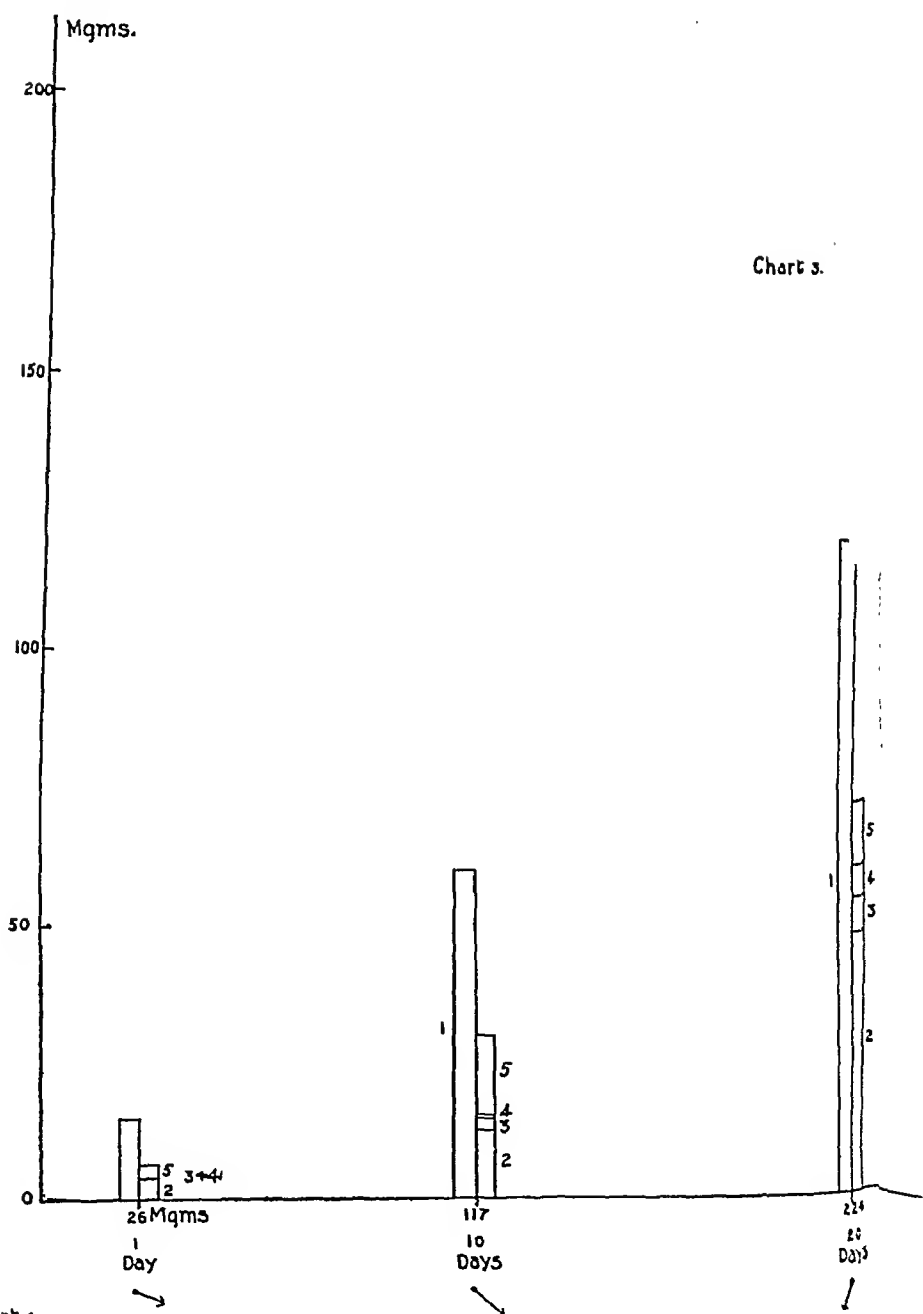


CHART 2. Shows the rate of increase of the different constituents of the brain of the albino rat at different ages, taking the amount of each constituent in the brain at birth as unity. (The ordinate shows how many times the weight of each constituent has increased over its amount at birth at the age plotted on the abscissa.)



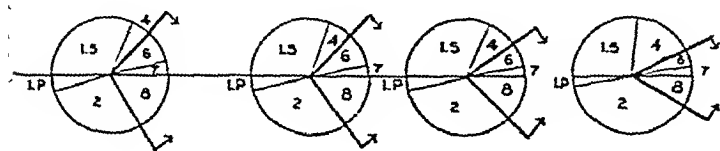
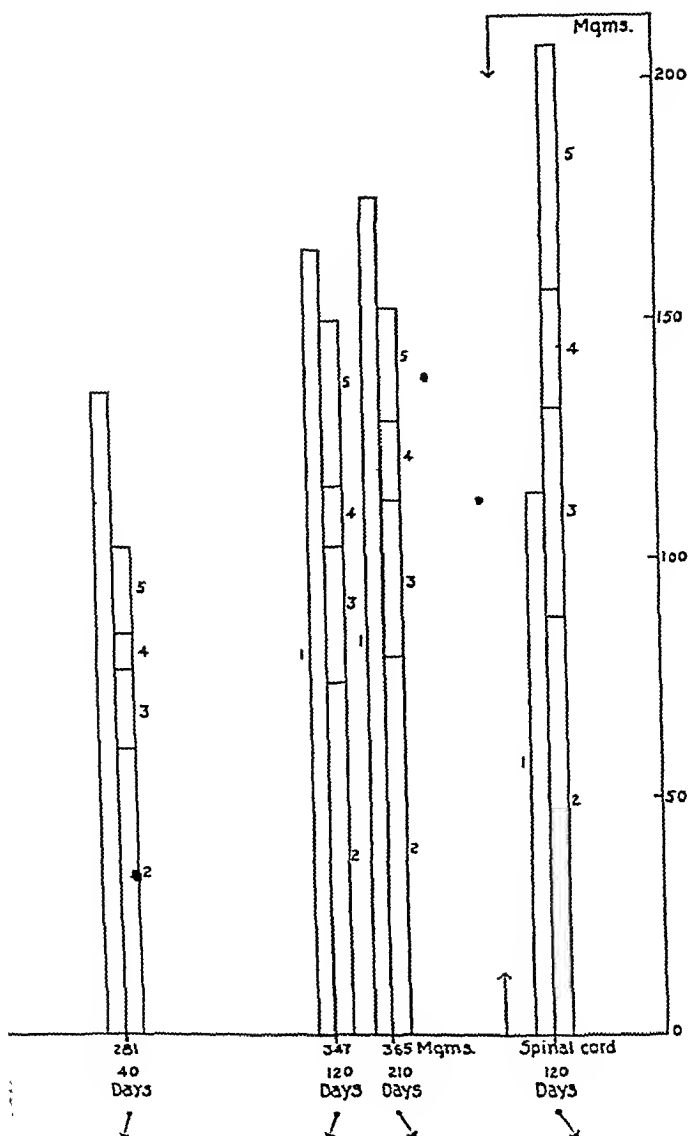


CHART 3. Shows, in absolute weight, the amounts of proteins and of lipoids in the brain of the albino rat at different ages. Based on Table IV. 1, Proteins; 2, Phosphatides; 3, Cerebrosides; 4, Sulphatides; 5, Undetermined lipoids (Cholesterol). The organic extractives and inorganic constituents are not included. For comparison the weights of the several constituents in the spinal cord of the albino rat at 120 days are also shown; the weight of the dry substance of the cord being taken as equal to that of the 120-day brain.

CHART 4. Shows, in the albino rat at different ages, the proportional values in segments of a circle for the sulphur combinations (above the equator) and for the phosphorus combinations (below the equator); with a further grouping into colloids and non-colloids. Based on Table IV. Again for comparison the proportional values for the sulphur and the phosphorus combinations in the spinal cord of the albino rat at 120 days are also given.

Sulphur combinations: 1, Proteins (protein sulphur); 4, Sulphatides (lipoid sulphur); 6, Neutral sulphur (proteic acids); 7, Inorganic sulphates.

Phosphorus combinations: 1, Proteins (protein phosphorus); 2, Phosphatides (lipoid phosphorus); 8, Organic and inorganic phosphates (water soluble phosphorus).

The colloids are represented by 1 and 4, of the sulphur combinations and 1 and 2 of the phosphorus combinations.

The non-colloids are represented by 6 and 7 of the sulphur combinations and by 8 of the phosphorus combinations.

teins are present in the brain in nearly equal proportions. For the sake of comparison, the corresponding values for the spinal cord at 120 days have been introduced into this chart.

To make easier the comparison between the relations of proteins and lipoids in the brain and in the spinal cord, it is assumed for the purposes of the chart that the dry weight of the cord is the same as that of the brain at 120 days. Since the cord contains a larger proportion of white matter than does the brain, we find that the lipoids in this case predominate over the proteins. This indicates that the chemical differentiation during the growth of the nervous system, as recorded in this paper, is largely concerned with the development of the medullated nerve fiber.

Chart 4 shows very strikingly the great decrease with advancing age in the non-colloidal contrasted with the corresponding increase in the colloidal sulphur and phosphorus compounds. Particular attention is called to the neutral sulphur which in the young, rapidly metabolizing tissue constitutes the greater proportion of the total sulphur, whereas it becomes extremely small at 210 days when growth metabolism is at an end. Evidently this fraction may, with reserve, be considered an index of growth metabolism. With advancing age the colloidal, less active, substances gradually crowd out the non-colloidal. This is in striking accord with the interesting suggestions of Child³³ that senescence is due to the accumulation of these colloidal solids, which interpose resistance to metabolism.

Finally we find the growth process characterized by a steady diminution in the proportions of water and an increase in the proportion of solids. This change is due not alone to medullation in the strict sense, since as Donaldson³⁴ has pointed out the decrease begins before medullation, between birth and ten days in the rat. He attributes it to a rapid growth of the axone at this time. Water and the proportion of neutral sulphur are therefore criteria of the youthfulness of tissue, while the increase of lipid sulphur (sulphatides) is a criterion of medullation.

³³ Child, C. M.: *Archiv. f. Entwicklungs-mechanik d. Organismen*, xxxi, p. 571, 1911.

³⁴ Donaldson, H. H.: *Journ. of Neurology and Psychology*, xx, p. 133, 1910.

SUMMARY.

The principal results of this study may be summarized as follows:

Well-marked and characteristic chemical changes occur in the rat-brain during its growth and these changes are obviously correlated with its anatomical differentiation.

The principal chemical changes noted are:

1. A general decrease in the per cent of water which is not due entirely to medullation since the decrease begins before medullation (Donaldson '10).

2. A diminution in the relative per cent of protein in the total solids due to the formation of a large amount of lipoid matter.

3. The lipoids which appear coincident with medullation and of which the development is *pari passu* with medullation are the cerebrosides and sulphatides. These, therefore, are chiefly found in the medullary sheaths.

4. There is a great outburst of phosphatide formation at the very beginning of medullation, but the phosphatides are present also in large amounts before medullation. The phosphatides are present, therefore, in the cells as well as the sheaths.

5. The extractives are present in largest amounts during fetal and early life when growth and metabolism are at a maximum. Particularly the water-soluble, organic sulphur compounds (neutral sulphur) diminish relatively with age, while the colloidal sulphur increases. The relations of the neutral sulphur may be interpreted, therefore, as indicating the intensity of metabolic activity.

6. The great increase of colloidal matter with age clearly indicates that this, in the form of supporting structures, constitutes a relatively inactive material which presumably serves to localize chemical processes. The accumulation of this material is probably one factor producing the general slowing of metabolism characteristic of senescence. This would thus become one cause of senescence as Child has suggested.

ON THE PRESENCE OF ADENASE IN THE HUMAN BODY.

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(Received for publication, July 8, 1913.)

The individuality of the two deaminizing enzymes concerned in purine metabolism, guanase and adenase, can be considered established. The former contention of Schittenhelm that one deaminizing ferment converts guanine into xanthine and adenine into hypoxanthine, can no longer hold, in the light of accumulated evidence of the ability of some organs to convert one of the purines without affecting the other. Of these two enzymes guanase is much more widely distributed in animal tissues. It is still a disputed question whether or not adenase occurs in the human body at all.

The method which has usually been applied in the study of purine enzymes is the subjection of purines *in vitro* to the action of extracts of animal tissues. How well this method represents the actual processes as they take place within the living organism, cannot be said. Such experiments should be supplemented where possible by a study of the fate of purines introduced within the living body, and experiments of this kind are recorded in the literature.

The latest investigations of the action upon adenine of extracts of human tissues may be summarized as follows: In an article published in 1907 Schittenhelm and Schmid¹ reported that added adenine was destroyed after seventeen days' incubation with extracts of human liver, intestine, and thymus, no purines at all being recovered, and that adenine was converted into hypoxanthine by extract of muscle and kidney, and that it was not attacked by extract of lung. In a later investigation Schittenhelm² revised his findings, obtaining evidence that adenine is converted into

¹ *Zeitschr. f. exp. Path. u. Ther.*, iv, pp. 424-431, 1907.

² *Zeitschr. f. physiol. Chem.*, lxiii, pp. 248-268, 1909.

hypoxanthine by extract of human lung, and to a less extent by extracts of kidney and intestine, inconclusive results being reached with liver and muscle. He asserts, in opposition to the contention of Jones and his co-workers, who in a long series of investigations have reached the conclusion that adenase is not present in the human body, that human tissues have in varying degree the ability of converting adenine into hypoxanthine. Winternitz and Jones³ found that adenine could be recovered quantitatively after treatment with extracts of human liver and spleen, and Miller and Jones⁴ found that adenase was not present in the spleen, liver, pancreas, kidney, or lung and claim that the enzyme is not contained in human tissue at all. Wells and Corper,⁵ on the other hand, found that extracts of fetuses of the five and six month stages converted adenine, but that the extract of a three months' fetus did not, and furthermore that adenine could not be recovered, while hypoxanthine was obtained in quantity, in the autolysis of a fetus of the sixth month. That this last, however, can be taken in another light than as indicating the presence of adenase, will be seen from the following:

It has been repeatedly proved that hypoxanthine is not necessarily a product of the action of adenase upon adenine, and may occur free in tissues containing no adenase. This Jones calls "preformed hypoxanthine." Leonard and Jones⁶ found that pig and rabbit muscle, in which adenase is not present, nevertheless contain large amounts of hypoxanthine, and further that hypoxanthine is always one of the products of autolysis of human spleen, although this tissue contains no adenase; that is, its extract will not convert adenine into hypoxanthine. After similar experience with other tissues Leonard and Jones and Vögtlin and Jones⁷ were led to believe that "preformed hypoxanthine" was present in all tissues. Straughn and Jones⁸ demonstrated its presence in yeast, which does not contain adenase. Wells and Long⁹ found that in the prolonged autolysis of human tumors which did not contain adenase, no adenine could be recovered, while great amounts of hypoxanthine were formed. Quite recently Amberg and Jones¹⁰ have shown that hypoxanthine may be formed in the splitting of nucleic acid by enzymes, without passing through the adenine stage, adenosine and inosine being the intermediate products.

Experiments on the fate of adenine fed to human beings are not so numerous. Krüger and Schmid¹¹ fed adenine to a man and found an

³ *Zeitschr. f. physiol. Chem.*, lx, pp. 180-190, 1909.

⁴ *Ibid.*, lxi, pp. 395-404, 1909.

⁵ *This Journal*, vi, pp. 469-482, 1909.

⁶ *Ibid.*, vi, pp. 453-460, 1909.

⁷ *Zeitschr. f. physiol., Chem.*, lxvi, pp. 250-256, 1910.

⁸ *This Journal*, vi, pp. 245-255, 1909.

⁹ *Zeitschr. f. Krebsforschung*, xii, pp. 598-611, 1913.

¹⁰ *Zeitschr. f. physiol. Chem.*, lxxiii, pp. 407-415, 1911.

¹¹ *Ibid.*, xxxiv, pp. 549-565, 1902.

increase in the elimination of uric acid corresponding to 41 per cent of the nitrogen of the adenine fed. They also noticed an increase of 3 per cent in the adenine of the urine. A similar increase in the purine output was not noticed when the other purines were fed. Brugsch and Schittenhelm,¹² in feeding purines to a gouty individual, found an increase of uric acid elimination of 50 per cent attributable to the adenine fed. Krüger and Solomon¹³ have published an analysis of 10,000 liters of urine in which 10.11 grams of xanthine, 8.50 grams of hypoxanthine and 3.54 grams of adenine, were found. From the low result of adenine they conclude that much of the ingested adenine was destroyed. Jones¹⁴ calls attention to the fact that by the method used (Neubauer's) hypoxanthine could be formed from adenine and carnine. Mendel and Lyman¹⁵ found that the ingestion of adenine in man caused a marked rise in the uric acid elimination, and a small but noticeable increase in the elimination of purine bases. These results would seem to indicate that when adenine is fed to man some is destroyed, uric acid resulting as the end product in its metabolism, while a portion is not affected, but is excreted as the free base.

In view of these facts the following experiments were performed to ascertain whether adenase could be demonstrated *in vitro* in human tissues, either by examination of individual organs or of extracts of entire fetuses.¹⁶

EXPERIMENTAL PART.

In the following experiments given amounts of adenine or one of its salts were added to weighed samples of ground tissue with 500 cc. of water, and the mixtures incubated in air-tight bottles at 38° for two weeks, toluene being used as antiseptic. In all cases where sufficient tissue was available, control experiments were made, in which adenine was incubated with the extract of the same amount of tissue after the mixture had been boiled one hour. The procedure used for the isolation of purines at the end of that time was the copper sulphate and sodium bisulphite method of Krüger and Solomon, the adenine being determined as picrate and the hypoxanthine as hypoxanthine silver nitrate. In a previous set of purine analyses it was noted that certain precautions were necessary in the precipitation of adenine picrate.

¹² *Zeitschr. f. exp. Path. u. Ther.*, v, pp. 215-226, 1903.

¹³ *Zeitschr. f. physiol. Chem.*, xxvi, p. 367, 1898.

¹⁴ *Ibid.*, lxxv, pp. 383-388, 1910.

¹⁵ *This Journal*, viii, pp. 115-143, 1910.

¹⁶ The materials used in this investigation were received from several Chicago Hospitals through the kindness of Drs. Jobling, Le Count, Gill, Henry and Davis.

Traces of mucilaginous substances from the tissue extracts may frequently be carried along in the analysis as far as the adenine stage, and these substances seem able to retard or even prevent the precipitation of adenine picrate. This difficulty can be overcome by reprecipitation of the purines with copper sulphate and sodium bisulphite, and treatment with picric acid of the cold filtrate after the decomposition of the copper purine compounds by hydrogen sulphide.

The liver, upon which the evidence in the literature with regard to the presence of adenase is contradictory, was first studied.

EXPERIMENT I. The liver used was from an accident case and was received a few hours after death. Microscopic examination showed it to be very fatty. 100 grams of the ground up tissue were added to a solution of 0.292 gram of adenine sulphate and the mixture kept, as in all the following experiments, at a temperature of 38°. It was very faintly acid, as were all the emulsions of this series of experiments. A boiled control, containing the same ingredients, was run at the same time. The figures for the purines added and those found by analysis after incubation, follow:

	ADDED		RECOVERED			
	Adenine sulphate	Calculated adenine	Adenine picrate	Calculated adenine	Hypo-xanthine Ag NO ₃	Calculated hypo-xanthine
Experiment.....	0.292	0.214	0.412	0.152	0.195	0.073
Control.....	0.292	0.214	0.442	0.164	0.124	0.046

EXPERIMENT II. The liver used, also from an accident case, was macroscopically and microscopically normal. 100 grams of tissue were used in the experiment and control. The results in this experiment, tabulated, were as follows:

	ADDED		RECOVERED			
	Adenine hydrochloride	Calculated adenine	Adenine picrate	Calculated adenine	Hypo-xanthine Ag NO ₃	Calculated hypo-xanthine
Experiment.....	0.230	0.170	0.294	0.109	0.194	0.073
Control.....	0.230	0.170	0.255	0.095	0.172	0.064

The adenine picrate separated out very slowly in this experiment even after reprecipitation of the purines by copper. Possibly a complete sep-

aration was not effected, in which case the hypoxanthine figures would be too high.

As other experiments have repeatedly shown that in the presence of adenase, adenine is completely converted into hypoxanthine in the amount used and time allowed above, and as in both cases the recovery of adenine (70 and 65 per cent) was about the same in the actual experiment as in the boiled controls, it may be concluded that the loss of adenine was due to experimental error and that adenase was not present. The hypoxanthine recovered represents the "preformed hypoxanthine" of the tissue used. The larger hypoxanthine recovery from the unboiled tissue can be explained as the result of the 14-day autolysis of the fresh tissue, it having been shown that in autolysis of tissues hypoxanthine is regularly formed even in the absence of adenase (see above and Amberg and Jones¹⁷).

As no work on the presence of adenase in the human placenta was found reported in the literature, the action of an extract of this organ upon adenine was studied:

EXPERIMENT III. (100 grams of tissue used.)

	ADDED		RECOVERED			
	Adenine sulphate	Calculated adenine	Adenine picrate	Calculated adenine	Hypoxanthine AgNO ₃	Calculated hypoxanthine
Experiment.....	0.215	0.161	0.329	0.122	0.102	0.038
Control.....	0.215	0.161	0.391	0.145	0.046	0.017

The inability of extract of placenta to destroy adenine is thus shown. It may be mentioned that the action of the placental extract upon guanine was studied at the same time and the presence of guanase demonstrated by the complete conversion of added guanine into xanthine.

The following experiments were made either with the individual organs or with uniform samples of the ground up tissue of entire human fetuses. As mentioned above Wells and Corper¹⁸ by the latter method obtained evidence of the presence of adenase in the bodies of fetuses above the fifth month.

¹⁷ *Loc. cit.*

¹⁸ *Loc. cit.*

EXPERIMENT IV. Male fetus, about the seventh or eighth month length 41 cm.; weight 1260 grams. Liver. (47.5 grams of tissue used.)

	ADDED		RECOVERED			
	Adenine sulphate	Calculated adenine	Adenine picrate	Calculated adenine	Hypo-xanthine Ag NO ₃	Calculated hypo-xanthine
Experiment.....	0.219	0.161	0.342	0.127	0.096	0.036

The rest of the fetus was ground up separately and the resulting emulsion thoroughly mixed. A 100-gram sample, representing all the organs of the body except the liver, was incubated for two weeks with a solution of 0.172 gram of adenine sulphate. A boiled control was run as usual. Very great difficulty and corresponding loss of purines due to the gelatinous material present were experienced in the filtration from tissue following the period of incubation. The use of aluminum cream and animal charcoal as coagulants was tried without much success. The mixtures were finally filtered on a hot funnel and the purine separation was made as usual. The solution representing the fresh tissue yielded no precipitate with picric acid on forty-eight hours' standing, while a precipitate of adenine picrate (m. p. 280°) came out readily in the boiled control. The figures for this experiment follow (100 grams of tissue were used):

	ADDED		RECOVERED			
	Adenine sulphate	Calculated adenine	Adenine picrate	Calculated adenine	Hypo-xanthine Ag NO ₃	Calculated hypo-xanthine
Experiment.....	0.172	0.126	0	0	0.123	0.045
Control.....	0.172	0.126	0.159	0.059	0.048	0.018

Adenase was evidently not present in the liver, adenine being recovered in quantity after treatment with the fresh tissue. The evidence with respect to the rest of the tissue is not conclusive. Adenase appeared to be present. Adenine was recovered from the boiled control, while no adenine could be recovered after treatment of its solution with 100 grams of the fresh tissue, but the quantity of hypoxanthine recovered was too small to be taken as representing with absolute certainty the action of adenase upon the adenine added, although the difficulties and consequent loss in analysis may account for the low recovery.

EXPERIMENT V. Female fetus about the fifth or sixth month; length 29 cm.; weight 490 grams. (The liver had been removed at the hospital.)

The body was ground up and uniformly mixed, and the action of a portion of the emulsion upon adenine tested as usual; 100 grams of tissue were used.

	ADDED		RECOVERED			
	Adenine hydrochloride	Calculated adenine	Adenine picrate	Calculated adenine	Hypoxanthine Ag NO ₃	Calculated hypoxanthine
Experiment.....	0.134	0.100	0.208	0.077	0.093	0.035
Control.....	0.134	0.100	0.251	0.093	0.036	0.013

As seen, adenine was recovered both from the experiment and from the boiled control.

EXPERIMENT VI. Female fetus; full term; length 52.7 cm.; weight 2190 grams. (The brain had been removed at the hospital.) The entire fetus was ground up and uniformly emulsionized as usual. 100 grams of tissue were used. The analytical data were:

	ADDED		RECOVERED			
	Adenine sulphate 2H ₂ O	Calculated adenine	Adenine picrate	Calculated adenine	Hypoxanthine Ag NO ₃	Calculated hypoxanthine
Experiment.....	0.261	0.160	0.317	0.118	0.120	0.045
Control.....	0.261	0.160	0.329	0.123	0.041	0.015

Clearly the samples of tissue used in the last two experiments were unable to attack adenine, practically as much of the latter being recovered from the fresh extracts as from the boiled controls.

EXPERIMENT VII. Female fetus; full term; weight 3760 grams. The entire fetus was ground up and two uniform samples were taken as usual, and incubated with solutions each containing 0.123 gram of adenine, one of which was boiled for control. After fourteen days the purine separation was made as usual. No adenine could be recovered by pieric acid precipitation in the solution representing the action of the fresh tissue, while adenine picrate settled out characteristically in the boiled control. The purines of this solution were then reprecipitated with copper sulphate and sodium bisulphite and the copper compounds decomposed as usual by hydrogen sulphide. Adenine could not be recovered from the clear, colorless filtrate from copper sulphide, even on treatment of the solution with pieric acid over night. So hypoxanthine was then determined with certain precautions. The purines were reprecipitated as the copper compounds and then as the silver compounds. The silver nitrate was next made in the usual manner by treatment of the silver purine compound with hot dilute nitric acid. A few crystals of urea were added to prevent the deaminizing action of any trace of nitrous acid in the nitric acid used, which was of the purest variety, upon any adenine possibly present. The

crystalline compound which settled out upon cooling weighed 0.181 gram, corresponding to 0.068 gram of hypoxanthine. This yield was of course low and for the following reasons: the purification was quite complete and attended by the usual slight, unavoidable losses at each stage, and furthermore the substance was crystallized from a rather large volume of water (150 cc.). It was next recovered as the free purine and crystallized as the hydrochloride. The latter was prepared in the anhydrous form and analyzed for nitrogen by the Kjeldahl method.

0.0272 gram contained 0.0091 gram N or 33.45 per cent. Theory for hypoxanthine hydrochloride, $C_5H_5N_4O \cdot HCl$, 32.48 per cent. As anhydrous adenine hydrochloride contains 40.8 per cent N there can be no question as to the identity of the recovered compound.

From the boiled control adenine was recovered quantitatively as shown in the following tabulation of the results of this experiment. 100 grams of tissue were used.

	ADDED		RECOVERED		
	Adenine	Adenine picrate	Calculated adenine	Hypoxanthine Ag NO ₃	Calculated hypoxanthine
Experiment.....	0.123	0	0	0.181	0.068
Control.....	0.123	0.322	0.119	0.056	0.021

The presence of adenase in the fetus used in this case seems to be demonstrated. Adenine was recovered quantitatively from a boiled control, while it could not be recovered at all from the fresh extract, its place being taken by hypoxanthine.

The finding of the enzyme in an extract of fetal tissue taken into consideration with the negative findings of Jones and his coworkers in the study of adult organs, suggested the possibility that adenase was present in the organ, well developed in the infant but atrophied in the adult, viz., the thymus. As mentioned above, Schittenhelm and Schmid¹⁹ reported the destruction of adenine by extracts of the human thymus. Accordingly this organ was examined for adenase, with negative result:

EXPERIMENT VIII. (2.5 grams thymus tissue used.)

ADDED		RECOVERED			
Adenine sulphate	Calculated adenine	Adenine picrate	Calculated adenine	Hypoxanthine Ag NO ₃	Calculated hypoxanthine
0.130	0.098	0.198	0.078	0.010	0.004

¹⁹ *Loc. cit.*

Thus adenase was not present. The result of Schittenhelm and Schmid was not confirmed.

Other fetuses were next obtained and after the removal of some of the organs for individual study, examined in the same manner as the preceding ones.

EXPERIMENT IX. Female fetus, about seventh month; length 33 cm.; weight 1140 grams.

Brain. (50 grams of tissue were used.)

	ADDED		RECOVERED			
	Adenine sulphate	Calculated adenine	Adenine picrate	Calculated adenine	Hypo-xanthine Ag NO ₃	Calculated hypo-xanthine
Experiment.....	0.199	0.145	0.367	0.136	0.035	0.013
Control.....	0.199	0.145	0.362	0.134	0.006	0.002

Bones. (50 grams, ground and mixed.)

	ADDED		RECOVERED			
	Adenine sulphate	Calculated adenine	Adenine picrate	Calculated adenine	Hypo-xanthine Ag NO ₃	Calculated hypo-xanthine
Experiment.....	0.199	0.145	0.242	0.090	0.114	0.043
Control.....	0.199	0.145	0.335	0.124	0.003	0.003

The other tissues were ground up and uniformly mixed. 100-gram samples of the uniform emulsion were taken as in the preceding experiments.

	ADDED		RECOVERED			
	Adenine sulphate	Calculated adenine	Adenine picrate	Calculated adenine	Hypo-xanthine Ag NO ₃	Calculated hypo-xanthine
Experiment.....	0 199	0 145	0	0	0 324	0 123
Control.....	0.199	0.145	0 236	0 088	0 086	0 032

Adenase was clearly absent from the brain and bone, adenine being recovered in quantity after treatment with the extract of each tissue. It appeared to be present however in the mixed mass of ground tissue representing all the other organs of the body. No adenine could be recovered after treatment with this tissue, its place being taken by hypoxanthine, while it was recovered in considerable quantity from the boiled control.

EXPERIMENT X. Male fetus; seventh to eighth month; length 40 cm.; weight, 1670 grams. The stomach, intestines, and pancreas, were removed, ground together, and tested for adenase in the usual manner. The results were as follows:

	ADDED		RECOVERED*			
	Adenine sulphate	Calculated adenine	Adenine picrate	Calculated adenine	Hypoxanthine Ag NO ₃	Calculated hypoxanthine
Experiment.....	0.147	0.107	0.184	0.068	0.036	0.014
Control.....	0.147	0.107	0.164	0.061	0.036	0.014

*An accident in manipulation lowered the recovery by about a fourth in each case.

All the remaining tissues were ground together and intimately mixed. Uniform samples of 100 grams were taken as usual:

	ADDED		RECOVERED			
	Adenine sulphate	Calculated adenine	Adenine picrate	Calculated adenine	Hypoxanthine Ag NO ₃	Calculated hypoxanthine
Experiment.....	0.147	0.107	0	0	0.306	0.115
Control.....	0.147	0.107	0.134	0.050	0.191	0.071

Apparently adenase was present in an emulsion of all the tissues other than the stomach, intestines, and pancreas—it could not be demonstrated in the latter tissues—for hypoxanthine and no adenine was recovered after treatment with the fresh tissue. The results are, however, not conclusive, for unfortunately a slight putrefactive odor was noted in the mixture after incubation, showing that some bacterial action had taken place in spite of the antiseptic added, so that the possibility of conversion by bacterial enzymes cannot be excluded. The recovery of adenine from the boiled control was for some unknown reason lower than usual.

The hypoxanthine fractions recovered from the tests with the entire fetuses in Experiments IX and X were put together, repurified, and the purine crystallized as the anhydrous hydrochloride.

Analysis (Kjeldahl).....32.71 per cent N.
Theory for hypoxanthine hydrochloride,
 $C_5H_5N_4O \cdot HCl$32.48 per cent N.

EXPERIMENT XI. Male fetus; full term; weight 3665 grams. The lungs, intestines, and spleen, were examined separately, with the results tabulated below:

Lungs. (40 grams of tissue used.)

ADDED	RECOVERED			
	Adenine picrate	Calculated adenine	Hypoxanthine Ag NO ₃	Calculated hypoxanthine
0.197	0.489	0.181	0.099	0.037

Intestines. (50 grams of tissue used.)

ADDED	RECOVERED			
	Adenine picrate	Calculated adenine	Hypoxanthine Ag NO ₃	Calculated hypoxanthine
0.197	0.465	0.172	0.058	0.023

Spleen. (10 grams of tissue used.)

ADDED	RECOVERED			
	Adenine picrate	Calculated adenine	Hypoxanthine Ag NO ₃	Calculated hypoxanthine
0.123	0.306	0.113	0.020	0.007

An emulsion was also made containing portions of all the other tissues of the body, which was examined for the presence of adenase in the usual manner: 100 grams of tissue were used.

	RECOVERED				
	ADDED Adenine	Adenine picrate	Calculated adenine	Hypoxanthine Ag NO ₃	Calculated hypoxanthine
Experiment.....	0.197	0.347	0.139	0.273	0.102
Control.....	0.197	0.542	0.200	0.061	0.024

Thus adenase was found to be absent from the lungs, intestines, and spleen, and its presence could not be demonstrated in an emulsion of the other tissues, added adenine being recovered in all cases. The amount of adenine recovered from the treatment with the emulsion was lower than that recovered from the boiled control and the hypoxanthine recovery was considerably higher.

However, this cannot safely be interpreted as a result of the action of adenase for the losses due to mechanical factors may show considerable variance in operations involving the use of the quantity of tissue here employed, and a high hypoxanthine recovery is a finding which was regularly observed in this series of experiments where 100 grams of tissue were allowed to autolyze for two weeks. This formation of hypoxanthine in the autolysis of tissues not containing adenase, as mentioned before, is explained by Amberg and Jones²⁰ as the result of a transformation of the adenine radicle of nucleic acid by way of other intermediate products than adenine, viz., adenosine and inosine, adenosine deaminase and inosine hydrolase being the enzymes concerned in the process.

Adenase was clearly absent from the lungs, intestines, and spleen, adenine being recovered quantitatively after treatment with the fresh tissue, a result in agreement with the findings of Miller and Jones.²¹ The results obtained with the mixed tissues of the fetuses, used in this investigation are, as seen, inconsistent. In Experiments IV, VII, IX, and X adenine added to an extract of the fresh thoroughly mixed tissue representing portions of the entire body other than those taken for separate examination, could not be recovered after two weeks' incubation, hypoxanthine being found in its place. These findings are in agreement with those of Wells and Corper²² in the case of fetuses of the five and six month stages. The negative result in Experiment V in which a five-month fetus was used may possibly be explained on the assumption that the enzyme had not yet developed. The experiments of Jones and Austrian,²³ Wells and Corper²⁴ and Mendel and Mitchell²⁵ show that the purine enzymes appear at different periods and it is reasonable to suppose that under certain conditions the appearance might be delayed. But the results of Experiments VI and XI in which the enzyme could not be demonstrated in the bodies of full term fetuses, seem hardly reconcilable

²⁰ *Loc. cit.*

²¹ *Loc. cit.*

²² *Loc. cit.*

²³ This *Journal*, iii, pp. 227-232, 1907.

²⁴ *Loc. cit.*

²⁵ *Amer. Journ. of Physiol.*, xx, pp. 81-96, 1907.

with those of Experiments IV, VII, IX, and X in which extracts of emulsionized fetuses converted adenine into hypoxanthine. (The result in Experiment X is open to question as explained in connection with that experiment.) It would seem that adenase is contained in the body of the human fetus, but that its location is not known, and whether because of its low concentration or for some other reason uniform samples from some emulsionized bodies may show its presence while others may not.

SUMMARY.

Adenase could not be demonstrated in the human adult liver, the placenta, or the fetal liver, brain, bone, thymus, stomach, intestines, pancreas, lungs, and spleen.

Evidence of its presence in the body of the human fetus was obtained, however, by treating adenine with 100-gram samples of thoroughly mixed tissues of the entire bodies in the case of four fetuses, while such evidence was lacking in the case of three other fetuses examined.

Hypoxanthine is always formed in the autolysis of human tissues, whether they contain adenase or not, a finding which signifies a transformation of the adenine radicle of nucleic acid by way of other intermediate products than adenine, probably through the action of adenosine deaminase and inosine hydrolase, as suggested in the case of similar findings in other tissues by Amberg and Jones.

GLYOXALASE. PART III.

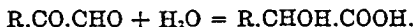
THE DISTRIBUTION OF THE ENZYME AND ITS RELATION TO THE PANCREAS.

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In two previous communications we have dealt with the preparation, mode of action and conditions for optimum activity of glyoxalase.¹ This enzyme converts α -ketonic aldehydes such as methyl and phenyl glyoxals into optically active lactic and mandelic acids:



We have already referred to the possible physiological significance in connection with the metabolism of carbohydrates.² In the present communication we wish to deal further with the distribution of the enzyme and particularly with the effect of the pancreas in inhibiting its action.

We have found no difficulty in obtaining highly active glyoxalase preparations from the blood, liver or muscle of a variety of animals, including man, dog, cat, calf, sheep, rabbit, fowl, toad, codfish, skate and oyster. On the other hand vegetable organisms, such as yeast and green plants, contain relatively less glyoxalase, and in some cases our experiments with plant material have given entirely negative results. In general it may be said that the distribution of the enzyme harmonizes with its supposed function.

Extracts of all animal tissues that we have examined have given definitely positive indications of the presence of glyoxalase with one exception, namely, the pancreas. Since blood cells contain much glyoxalase the positive findings in the case of some organs

¹ This *Journal*, xiv, pp. 155, and 423, 1913.

² *Ibid.*, xiv, p. 555, 1913.

may possibly be due to unavoidable contamination but in most cases the glyoxalase is certainly present in the tissue cells. Glyoxalase is absent from urine and bile. *Further experiments showed that pancreatic tissue, aqueous extracts of the pancreas and pancreatic juice not only contain no glyoxalase but contain a thermolabile substance which exerts an intense inhibitory action on glyoxalase derived from other sources.*

We have observed the inhibitory action of the pancreas on glyoxalase in every animal species that we have examined, including man, dog, cat, rabbit, fowl and toad. In view of the known connection of the pancreas with carbohydrate metabolism it appeared desirable to study the relation between the pancreas and glyoxalase in some detail. The results which we have obtained thus far may be summarized as follows:

1. The inhibitory action of the pancreas of one species of animal is not limited to glyoxalase derived from the same species.

2. The inhibitory substance, which for convenience we may call antiglyoxalase, is found not only in extracts of the gland but is present in pancreatic juice obtained from dogs either by fistula or as the result of the injection of secretin. It is present in commercial trypsin and pancreatin preparations and may be preserved in the dry state indefinitely. A pancreas preparation more than eight years old was found to contain much antiglyoxalase.

3. Antiglyoxalase is destroyed by heating for ten minutes at 85°. It is slowly destroyed by digestion with weak hydrochloric acid ($\frac{1}{1000}$) much as trypsin is. The action of weak alkali (1 per cent sodium carbonate) is much less injurious.

4. Relatively small amounts of pancreas extract can inhibit the action of large amounts of active glyoxalase. Thus 0.025 gram of a pancreatin preparation containing much inert material or less than 2 cc. of pancreatic juice may completely inhibit the action of a glyoxalase solution which otherwise would be capable of forming several grams of lactic acid.

5. By observing the effect of varying amounts of antiglyoxalase during different intervals of time upon glyoxalase we have found that the inactivation of the latter is not instantaneous but is a function of the time. It is possible that antiglyoxalase is an enzyme accelerating the normal decomposition of glyoxalase and all the facts are in agreement with this hypothesis, but they could

be equally well reconciled with the view that the inactivation of glyoxalase is brought about by simple direct union with anti-glyoxalase without catalytic action. We have been unable as yet to settle this question definitely.

6. The inhibitory action of the pancreas upon glyoxalase is not due to trypsin, lipase or diastase. An antiglyoxalase solution may be freed from trypsin by digestion with sodium carbonate and the resulting solution which is entirely without digestive action on proteins may still possess its inhibitory action upon glyoxalase. Moreover the vegetable proteoclastic enzyme papayin is entirely without action upon glyoxalase. Neither lipase nor diastase apparently are responsible for the action of antiglyoxalase, since liver extracts containing much lipase, and salivary diastase have no action upon glyoxalase.

7. Saliva, extracts of gastric mucosa from the middle and cardiac end of the stomach, blood serum or small amounts of bile are without action on glyoxalase. Extracts of duodenal mucosa on the other hand have a pronounced inhibitory effect, but it is probable that this is due to antiglyoxalase of pancreatic origin.

It is noteworthy that the so-called "pyloric caeca" of the cod-fish which were long supposed to be homologous with the pancreas not only contain no antiglyoxalase but contain a small amount of glyoxalase. It is now generally recognized by biologists that the pyloric caeca have nothing to do with the pancreas which in the adult is extremely inconspicuous and liable to escape observation. It is interesting that the chemical and morphological findings should harmonize so satisfactorily.³

While the pancreas contains no glyoxalase we find that the thyroid of the dog yields a very active preparation. We propose examining the pituitary and suprarenal glands as soon as we are able to secure suitable material.

In view of the relation existing between the pancreas and glyoxalase, and the function of both in connection with carbohydrate metabolism it appeared desirable to examine tissues of diabetic and other pathological organisms with regard to their glyoxalase. We find that glyoxalase is present in human blood from diabetics and in blood and liver from diabetic dogs which have had their

³ Professor E. B. Wilson was kind enough to give us advice upon the biological matters.

pancreas removed ten days previously. Thus far our results appear to show that while the glyoxalase of the blood of glycosuric dogs is not changed from normal, the blood and tissues of human diabetics and of depancreatized dogs have somewhat less than the normal activity. It is curious that the removal from an animal of the organ which exerts so profound an inhibitory effect upon the action of glyoxalase should be followed by a decrease rather than an increase in the activity of the latter enzyme. It would be possible to indulge in many speculations as to the relation of glyoxalase to pancreatic diabetes. But the conditions especially in the case of these experiments with pathological material are very complicated and before we can draw any satisfactory conclusions it will be necessary to study the problem with better methods than we have at present been able to apply. We hope to return to these experiments later.

The opportunity for making the experiments with depancreatized dogs as well as an experiment with pancreatic juice and a number of others that are not reported in this paper, was afforded us by the kindly coöperation of Professors Richards and Sweet of the University of Pennsylvania. We wish to record our grateful appreciation of their generous assistance.

EXPERIMENTAL.

In all the experiments recorded in this paper we have made use of phenyl glyoxal as substrate rather than methyl glyoxal. We have satisfied ourselves that the action of glyoxalase on the two glyoxals is essentially similar, but the use of phenyl glyoxal has certain important advantages. In the first place the mandelic acid which is formed from it, is easily extracted by ether and crystallizes readily. In every case in which we record a positive glyoxalase reaction the mandelic acid has been isolated in clean crystalline form. The high optical activity of mandelic acid as compared with the low rotation of lactic acid is also a great advantage. For each separate digestion we have used 0.2 gram of phenyl glyoxal.

The tissue extracts were in almost all cases prepared by stirring one part of minced tissue with five parts of water and straining through muslin after one hour. The use of plain water rather than saline is a distinct improvement since it effects a much

better extraction of the mass and yields solutions with greater enzyme activity. Blood cells, leucocytes, etc., should not be washed with saline as much glyoxalase is lost in the process.

In most of the experiments we have avoided the use of toluene or have used it in limited amount since it has a decidedly injurious effect upon glyoxalase (Section V). By using sterile tissues taken directly from the animal the use of antiseptics is unnecessary. Moderate bacterial contamination such as may occur after short periods of digestion is entirely without effect on the action of glyoxalase and moreover the addition of phenyl glyoxal appears to favor asepsis. In every case precipitated chalk was used to maintain approximate neutrality.

The digestion mixtures were analyzed precisely as described in our former paper¹ except that the heating in the water bath was limited to three minutes and the mandelic acid was extracted by shaking with four successive quantities of ether. The residual mandelic acid crystals were dissolved in 10 cc. of water, treated with a pinch of charcoal if necessary and then examined in a 2 dm. tube in the polarimeter. Subsequently the acidity was determined by titrating 5 cc. with decinormal caustic soda. In the following records of experiments the rotations given are those actually observed in the 2 dm. tube, while the acidities represent the number of cubic centimeters of alkali required to neutralize the 10 cc. of mandelic acid solution. In general it is advisable to judge of the enzyme activity rather by the rotation of the mandelic acid than by the acidity since the organ extracts themselves yield relatively considerable amounts of acid so that the observed acidities are always too high. Special blank experiments have repeatedly shown that under the existing conditions no optically active substance other than mandelic acid occurs in the extracts in sufficient amount to give a significant rotation.

I. Distribution of glyoxalase.

In each experiment, unless otherwise stated, 50 cc. of strained 20 per cent tissue extract or 5 cc. of blood were allowed to act on 0.2 gram of phenyl glyoxal in the presence of chalk for about 20 hours.

¹ This *Journal*, xiv, p. 427, 1913.

ANIMAL	TISSUE	ACIDITY	ROTATION OF MANDELIC ACID	PRESENCE OF GLYOXALASE
		cc.	deg. C	
Man....	Blood.....	2.3 to 3.8	-1.03 to -1.30	+
	Pancreas.....	0.5	-0.02	-
	Urine (150 cc.).....	2.2	0	-
	Muscle.....	3.0 to 3.4	-0.45 to -1.13	+
	Liver.....	6.0 to 8.8	-2.3 to -2.58	+
	Heart muscle.....	already reported		+
	Kidney.....	already reported		+
	Brain.....	already reported		+
	Lung.....	already reported		+
Dog....	Thyroid.....	8.2	-1.65	+
	Gastric mucosa.....	4.7	-0.95	+
	Duodenal mucosa.....	1.3	-0.12	?
	Blood.....	3.6	-0.68 to -1.35	+
	Blood serum.....	1.4	0 to +0.05	-
	Bile.....	1.2	0	-
	Pancreas.....	1.4 to 2.0	0 to +0.05	-
	Pancreatic juice (20 cc.).....	1.2	-0.03	-
	Liver.....	7.2	-2.02	+
Calf....	Pancreas.....	1.2	+0.05	-
	Bile.....	1.3	+0.03	-
Sheep....	Blood.....	3.2	-0.83	+
	Muscle.....	6.6	-2.22	+
Cat....	Liver.....	8.3	-2.60	+
	Blood.....	1.9	-0.33	+
	Pancreas.....	1.2	-0.08	-
Rabbit..	Liver, muscle.....	8.4	-2.40	+
	Blood.....	3.0	-0.75	+
Fowl....	Liver.....	7.6	-2.51	+
	Muscle.....	6.9	-2.80	+
	Blood.....	5.4	-2.42	+
	Pancreas.....	1.6	-0.05	-
Codfish..	Liver.....	1.6	-0.35	+
	Muscle.....	1.0	-0.20	+
	Pyloric caeca.....	1.4	-0.17	+
Skate....	Muscle.....	2.6	-0.83	+
Toad....	Liver.....	8.0	-2.57	+

II. The effect of adding extracts of pancreas on the action of glyoxalase.

The experiments recorded in this section were made essentially in the same manner as those in Section I. Only a few of the experiments which were carried out are recorded as they all gave

substantially similar results. A variety of pancreatic extracts and commercial pancreatin preparations were used without disclosing any marked differences. The column marked "time" in the following table indicates the time during which the added pancreas preparation was allowed to act on the glyoxalase before adding phenyl glyoxal. Where no entry is made it is to be understood that phenyl glyoxal was added immediately. In each case 50 cc. of glyoxalase solution, 0.2 gram phenyl glyoxal, and excess of precipitated chalk were used.

ANIMAL	TISSUE	ADDED SUBSTANCE	TIME	ROTATION OF MAN- DELIC ACID	CONCLUSION
			hours	deg. C	
Dog....	Muscle..	—	—	-1.07	+
		Pancreas, 50 cc.	—	+0.05	Inhibition
		Pancreas, 50 cc.	—	-0.13	Inhibition
Sheep...	Blood...	—	—	-0.83	+
		Ox pancreas, 100 cc.	—	+0.03	Inhibition
		—	—	-2.8	+
Fowl...	Muscle and Liver	Fowl pancreas, 0.75 gram	1	-1.88	Partial inhibition
		Fowl pancreas, 0.75 gram heated to 85°	1	-2.65	No inhibition
		Pancreatin, 0.2 gram	1	-0.08	Inhibition
		Pancreatin, 0.05 gram	1	-1.55	Partial inhibition
		Pancreatin, 0.025 gram	1	-1.88	Partial inhibition
		Pancreatin, 0.2 gram heated to 85°		-2.75	No inhibition
		—	—	-2.6	+
Cat....	Muscle and Liver	Pancreatin, 0.2 gram	1	-0.32	Partial inhibition
		Cat pancreas, 3 grams	1	-0.08	Inhibition
Toad...	Liver....	—	—	-2.57	+
		Toad pancreas, 0.5 gram	3	-0.40	Partial inhibition

III. The action of pancreatic juice on glyoxalase.

Two sets of experiments with pancreatic juice were made. In the first of these it was found that as little as 2 cc. of pancreatic juice obtained by secretin injection when acting for two hours on a glyoxalase solution completely inhibited the action of the enzyme. In the second experiment the juice which was obtained from a fistula was added to the glyoxalase simultaneously with the phenyl glyoxal. In this case the addition of a larger amount was necessary to secure inhibition. Heated juice was without adverse effect. Fifty cc. of glyoxalase solution, 0.2 gram of phenyl glyoxal, and excess of chalk were used in each experiment.

SOURCE OF GLYOXALASE	PANCREATIC JUICE	TIME BEFORE ADDING PHENYL GLYOXAL	ROTATION OF MANDELIC ACID	EFFECT
	cc.	hours	deg. C	
Heart and tongue of dog.....	—	—	-0.45	
	2	2	-0.07	Inhibition
	5	2	-0.03	Inhibition
	10	2	+0.05	Inhibition
Skeletal muscle of dog.....	—	—	-1.13	
	2	—	-1.28	No inhibition
	20	—	-0.25	Marked inhibition
	2	—		tion
	(heated to 85°)	—	-1.38	No inhibition
	20 (heated to 85°)	—	-1.68	No inhibition

IV. The inactivation of glyoxalase by small amounts of antiglyoxalase acting for varying lengths of time.

Experiments were made to determine the rate of inactivation of glyoxalase by small amounts of antiglyoxalase with a view to gaining insight into the nature of the inhibitory substance. A small quantity of "pancreatin," suspended in a known volume of water, was added to a 20 per cent glyoxalase extract. The mixture was then divided into a number of equal parts which were placed in flasks containing chalk suspension. To one of these solutions phenyl glyoxal was immediately added and all were placed in the incubator at 37°C. After suitable periods

phenyl glyoxal was added to the contents of the other flasks, so that eventually a series of experiments was obtained in which the relative activities of equal amounts of glyoxalase after varying periods of incubation with identical quantities of antiglyoxalase were determined. Control experiments were made by following simultaneously the loss of activity of corresponding amounts of the original glyoxalase extract at incubator temperature.

In Experiment I, five flasks, each containing 50 cc. of 20 per cent glyoxalase extract from dog's liver, 0.05 gram of pancreatin and precipitated chalk were prepared. To one of these 0.2 gram of phenyl glyoxal was added immediately and all were placed in the incubator at 35°C. After one, three, five and seven hours a similar quantity of phenyl glyoxal was added to each of the other flasks respectively. Control experiments with 50 cc. of liver extract, fresh and after incubation for seven hours, were made.

Experiment II was carried out similarly to Experiment I. A 20 per cent extract of cat's muscle and liver was employed, and to each 50 cc. of this preparation 0.035 gram of "pancreatin" was used. Phenyl glyoxal (0.2 gram) was added at the beginning and after every hour for six hours. Three control experiments were made with fresh and incubated glyoxalase extract (50 cc.).

All solutions were incubated for about twenty hours after the addition of phenyl glyoxal and then worked up for mandelic acid in the usual way.

The following table gives the results of the two experiments.

EXP.	TIME	ROTATION OF MANDELIC ACID FROM	
		Glyoxalase-pancreatin mixture	Glyoxalase extract
	Hours	deg. C	deg. C
I.....	0	-2.38	-2.58
	1	-1.9	
	3	-0.45	
	5	0	
	7	0	-1.15
II.....	0	-1.63	-2.22
	1	-1.27	
	2	-0.47	
	3	-0.17	-1.73
	4	-0.10	
	5	0	
	6	+0.05	-1.3

The mandelic acid residues were dissolved in 10 cc. of water and examined in a 2 dm. tube. The rotations are those actually observed. The time of incubation before the addition of phenyl glyoxal is recorded.

V. The effect of adding substances other than pancreas upon the action of glyoxalase.

These experiments were carried out in precisely the same manner as those in Section II. They show that saliva, gastric mucosa, blood serum and digestion with papayin have no effect upon glyoxalase. In two experiments with bile no inhibition was noted, in the third a marked decrease in the action of glyoxalase was noted, but we are inclined to attribute this to precipitation caused by the very concentrated bile interfering with the action of the

SOURCE OF GLYOXALASE	ADDITION	TIME BEFORE ADDING PHENYL GLYOXAL	ROTATION OF MANDELIC ACID	CONCLUSION
		hours	degrees	
Fowl....	—	—	-2.88	
	10 cc. Saliva	3	-2.98	No inhibition
	—	—	-0.45	
Dog.....	Gastric mucosa, 5 grams	3	-1.75	No inhibition
	—	—	-2.30	
	Duodenal mucosa, 40 cc.	3	-0.33	Partial inhibition
Cat.....	—	—	-2.6	
	Duodenal mucosa, 37 cc.	1	-0.45	Partial inhibition
	—	—	-2.30	
Dog.....	Dog bile, 2 cc.	3	-2.28	No inhibition
	Dog bile, 3 cc.	3	-2.25	No inhibition
Cat.....	Cat bile, 3 cc.	3	-0.13	Inhibition (?)
	—	—	-2.20	
Fowl....	Papayin, 0.2 gram	1	-2.57	No inhibition
	Papayin, 0.2 gram	24	-2.45	No inhibition
	—	—	-1.35	
Dog.....	Dog serum, 20 cc.	—	-1.44	No inhibition
	Toluene, 2 cc.	2½	-0.08	Marked inhibition
	Toluene, 2 cc.	3	-0.12	Marked inhibition

chalk used for neutralization. The inhibitory action of duodenal mucosa extracts and the destruction of glyoxalase by toluene are clearly shown. In all cases the glyoxalase was obtained from the liver and skeletal muscles of the animals indicated except in the experiments with toluene when blood was used.

VI. *The separation of antiglyoxalase from trypsin.*

Experiments were made to remove trypsin without destroying antiglyoxalase by digesting an active pancreatin preparation with dilute acid or alkali. The experiments with alkali were successful while digestion with acid ($\frac{N}{10}$ hydrochloric acid) gave less favorable results since the antiglyoxalase was partly destroyed before all the trypsin was removed.

Pancreatin (0.2 gram) containing much antiglyoxalase was digested with 10 cc. of 1 per cent sodium carbonate solution at 37° for four, twenty and twenty-four hours respectively. After four hours' digestion the pancreatin had very little or no action on coagulated egg white and the trypsin was entirely destroyed with certainty in the case of the longer digestions. Each pancreatin preparation was added to 50 cc. of an active glyoxalase solution and digested for three hours, when 0.2 gram phenyl glyoxal and precipitated chalk were added and the mixtures incubated for twenty-four hours. The rotations (2 dm. tube) of the mandelic acid extract (10 cc.) were as follows:

(1) Glyoxalase + no pancreatin.....	-2.22°
(2) Glyoxalase + pancreatin digested 4 hours.....	-0.05°
(3) Glyoxalase + pancreatin digested 20 hours.....	+0.03°
(1) Glyoxalase + no pancreatin.....	-3.05°
(2) Glyoxalase + pancreatin digested 24 hours.....	-0.73°

The results indicate clearly that trypsin may be removed from pancreatin without destroying the action of antiglyoxalase.

VII. *Glyoxalase in diabetic tissues.*

These experiments are fragmentary and can only be regarded as preliminary to a more extended study. The human material was obtained for us by Dr. N. W. Janney of the Montefiore Home while, as already mentioned, we owe the material from depan-

creatized dogs to the kindness of Professors Richards and Sweet. The human diabetic blood was obtained from only moderately severe cases. The liver and pancreas were obtained at post mortem about twenty-four hours after death. The absence of glyoxalase from the liver cannot be regarded as significant although the enzyme does not usually disappear after death with great rapidity. The blood and liver from depancreatized dogs were obtained from nine to ten days after operation by Professor Sweet. In each case 5 cc. of blood or 50 cc. of 20 per cent tissue extract and 0.2 gram of phenyl glyoxal were used. The mixtures were analyzed as in previous experiments.

TISSUE	ACIDITY	ROTATION OF MANDELIC ACID	GLYOXALASE
		<i>deg. C</i>	
Diabetic human blood....	2.2	-0.50	+
Diabetic human blood....	2.8	-0.92	+
Diabetic human blood....	2.6	-0.83	+
Diabetic human liver....	2.2	+0.08	-
Diabetic human pancreas	0.5	-0.02	-
Depancreatized dog blood	1.7	-0.45	+
Depancreatized dog liver.	4.6	-0.93	+
Phlorhizin dog blood.....	3.2	-1.17	+

ON THE ACTION OF LEUCOCYTES AND OTHER TISSUES ON *dl*-ALANINE.

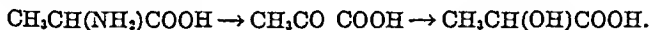
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The observations on the action of leucocytes and other tissues under aseptic conditions on hexoses made it possible to disclose the mechanisms of lactic acid formations from glucose.¹ The fact that *d*-lactic acid was formed regardless of the configuration of the hexose employed in the experiments forced the conclusion that methyl glyoxal was the immediate precursor of lactic acid.

It is generally accepted principally on the basis of the work of Otto Neubauer² that the conversion of amino-acids into hydroxy-acids is brought about by a process very analogous to that for formation of lactic acid from methyl glyoxal. Thus, for instance, alanine is transformed into lactic acid by passing through the stage of pyruvic acid.



This contention found support in the observations of Lusk and Ringer.³ These authors fed phlorhizinized dogs on *dl*-alanine and noted a complete conversion of the substance into *d*-glucose. Very recently this view was accepted on theoretical considerations by Dakin and Dudley.⁴ However, in the attempt to corroborate this view experimentally not all writers came to a close agreement. For instance, Paul Mayer,⁵ feeding phlorhizinized dogs on pyruvic acid failed to note a conversion of the substance into glucose. On the other hand, Dakin and Dudley, and Ringer,⁶ obtained a positive result. However, they noted that quantitatively the yield of sugar from pyruvic acid was smaller

¹ Levene and Meyer: this *Journal*, xiv, pp. 149, 551, 1913.

² Neubauer: *Deutsch. Arch. f. klin. Med.*, xcv, p. 211, 1909.

³ Ringer and Lusk: *Zeitschr. f. physiol. Chem.*, lxvi, p. 106, 1910.

⁴ Dakin and Dudley: this *Journal*, xv, p. 127, 1913.

⁵ Mayer: *Biochem. Zeitschr.*, xlix, p. 486, 1913.

⁶ Ringer: this *Journal*, xv, p. 145, 1913.

than that from either alanine or from lactic acid. Hence, it was desirable if possible to find a simpler method of testing the theory of the mechanism of lactic acid formation from alanine. Encouraged by the results of the experiments on the action of leucocytes on glucose,⁷ we attempted to test the action of leucocytes on the various optical isomers of alanine. It was thought that if pyruvic acid is the precursor of lactic acid then the same *d*-lactic acid might be obtained regardless of the nature of the alanine employed in the experiment. It was taken for granted that leucocytes possessed the power to deaminize alanine since it is generally accepted that the power of deaminization of amino-acids belongs to all tissues. The experiment was begun by the employment of *dl*-alanine. It was a surprise to find that under aseptic conditions leucocytes remained without perceptible action on *dl*-alanine. An attempt was therefore made to test the action of other tissues under aseptic conditions on *dl*-alanine. For this purpose the kidney was employed as previous experience had shown that the kidneys were the most suitable material that could be obtained in absolutely aseptic condition. However, the results of the latter experiments were identical with the results of the leucocyte experiments, namely, they showed that no perceptible deaminization took place.

Since these two series of experiments seemed not to harmonize with the generally accepted view on the deaminizing action of the tissues on amino-acids, it was thought that a considerable degree of autolysis of the tissues is required in order to bring their deaminizing action into play. For this reason experiments were performed with tissues in the presence of antiseptics. Kidney and liver were used in the experiments. However, when sufficient antiseptic was added to entirely prevent bacterial contamination no evidence was found of the deaminization of alanine in experiments that lasted from one to three weeks.

It is seen from this that alanine is not so readily deaminized as one was inclined to expect on the basis of the work of Lang⁸ and other observers. Whether or not this is true in regard to other amino-acids remains to be established. In a recent article on deaminization by Gertrude D. Bostock,⁹ from Cathcart's labo-

⁷ Levene and Meyer: this *Journal*, xi, p. 361, 1912; xii, p. 265, 1912.

⁸ Lang: *Hofmeister's Beiträge*, v, p. 321, 1904.

⁹ Bostock: *Biochem. Journ.*, vi, p. 48, 1912.

ratory, a statement is made that alanine behaves differently from glycocoll on treatment with liver and intestinal mucosa, but the alanine experiments are not reported in detail. We hope to repeat our observations on alanine on a series of other amino-acids.

EXPERIMENTAL.

Tissues. The kidney and liver used in these experiments were obtained from dogs which had been killed by exsanguination from the carotid while in ether narcosis. The tissues were hashed in a meat chopper and immediately weighed and minced with the alanine and phosphate solutions as described below. The leucocytes were obtained aseptically from the pleural cavity of dogs injected with turpentine according to the method previously described.¹⁰ In one experiment the kidneys of a rabbit removed aseptically were used.

Solutions. *dl*-Alanine in approximately 2 per cent concentration in a 1 per cent Henderson phosphate mixture was used in all experiments, excepting the leucocyte and aseptic kidney experiment. Toluene was used as antiseptic. The mixtures for each experiment were made up as follows:

Kidney.

1. 20.0 grams tissue suspended in 200 cc. alanine solution.
2. 5.0 grams tissue suspended in 50 cc. alanine solution.
3. 5.0 grams tissue suspended in 50 cc. phosphate solution.
4. 5.0 grams tissue suspended in 50 cc. phosphate solution.

Liver.

1. 40.0 grams tissue suspended in 200 cc. alanine solution.
2. 10.0 grams tissue suspended in 50 cc. alanine solution.
3. 10.0 grams tissue suspended in 50 cc. phosphate solution.
4. 10.0 grams tissue suspended in 50 cc. phosphate solution.

Leucocytes.

Approximately equal portions of leucocytes were mixed with

1. 50 cc. alanine solution.
2. 50 cc. alanine solution.
3. 50 cc. phosphate solution.
4. 50 cc. phosphate solution.

In the aseptic kidney experiment only two suspensions were prepared; each minced kidney was suspended in 50 cc. alanine

¹⁰ Levene and Meyer: this *Journal*, xi, p. 365, 1912.

solution. In each instance, suspensions 2 and 4 were coagulated immediately after mixing; 1 and 3 were set aside at 37° for from one to three weeks.

Methods of analysis. The contents of each flask were brought to a boil, coagulated and then poured into an excess of absolute alcohol to which was added sufficient 5 per cent alcoholic zinc chloride solution to completely precipitate the protein. This was filtered and the precipitate washed and extracted with hot water, and the filtrate and washing distilled in vacuum to completely remove the alcohol, which was only accomplished by repeating the distillation several times after addition of water. The residue was taken up in water and made up to a definite volume, usually twice that of the original, 50 cc. made up to 100 cc. 10 cc. of this solution were used for a total nitrogen determination, by the Kjeldahl method, 25 cc. for an ammonia determination, and, after removal of the ammonia, for an amino nitrogen determination by the Van Slyke method, as described in detail in a previous communication from this laboratory.

Bacteriological control. Aerobic and anaerobic cultures were made of the aseptic mixtures after incubation and smears made of the mixtures with toluene. There was no bacterial contamination. The bacterial examination was made by Dr. H. L. Amoss, and we take this occasion to express our appreciation of his assistance.

Analytical results.

The amino nitrogen determinations are here reported in detail. The results of the total nitrogen and ammonia nitrogen determinations are included in the appended tables.

Experiment I. Kidney tissue.

	ORIGINAL SOLUTION USED	ORG. VOLUME (conc.)	TEMPER- ATURE	PRESSURE	TIME	N	GRAMS PER 100 cc.
	cc.	cc.	°C.	mm.	min.	mgm.	
Alanine solution:							
Before.....	5	26.20	20	751	4	14.74	0.295
After three weeks.....	5	29.20	24	758	4	16.25	0.325
Phosphate solution:							
Before.....	5	1.30	24	760	4	0.78	0.015
After three weeks.....	5	3.30	24	758	4	2.00	0.040

Experiment II. Kidney tissue.

	ORIGINAL SOLUTION USED	GAS VOLUME (corr.)	TEMPER- ATURE	PRESSURE	TIME	N	GRAMS PER 100 CC.
	cc.	cc.	°C.	mm.	min.	mgm.	
Alanine solution:							
Before.....	5	28.00	18	760	4	16.10	0.322
After one week.....	5	30.30	20	758	4	17.30	0.346
Phosphate solution:							
Before.....	5	0.70	18	766	4	0.40	0.008
After one week.....	5	2.80	20	758	4	1.40	0.028

Experiment III. Liver tissue.

Alanine solution:							
Before.....	5	27.90	24	760	4	15.60	0.310
After three weeks.....	5	32.20	24	758	4	18.10	0.362
Phosphate solution:							
Before.....	5	2.20	24	760	4	1.28	0.026
After three weeks.....	5	6.20	24	758	4	3.44	0.068

Experiment IV. Liver tissue.

Alanine solution:							
Before.....	5	27.80	18	760	4	16.10	0.322
After one week.....	5	29.10	20	758	4	16.50	0.330
Phosphate solution:							
Before.....	5	1.00	18	766	4	0.58	0.012
After one week.....	5	3.30	20	758	4	1.87	0.037

Experiment V. Leucocytes.

Alanine solution:							
Before.....	2.5	14.75	22	760	4	8.31	0.332
After three weeks.....	2.5	17.60	20	758	4	19.90	0.398
Phosphate solution:							
Before.....	2.5	0.20	22	760	4	0.113	0.004
After three weeks.....	2.5	3.20	20	758	4	1.81	0.073

Experiment VI. Aseptic kidney.

Alanine solution:							
Before.....	2	11.70	20	760	4	6.65	0.332
After three days.....	2	11.70	20	758	4	6.64	0.332

*Experiments with kidney tissue and alanine.**With toluene.*

	AMMONIA N PER CENT			AMINO N PER CENT			TOTAL N PER CENT		
	Before	After	Diff.	Before	After	Diff.	Before	After	Diff.
I. Three weeks' incubation:									
Tissue and alanine.....	0.003	0.008	0.005	0.295	0.325	0.030	0.350	0.400	0.050
Tissue and phosphate solution.....	0.003	0.006	0.003	0.015	0.040	0.025	0.056	0.100	0.044
II. One-week incubation:									
Tissue and alanine.....	0.004	0.006	0.002	0.322	0.346	0.024	0.336	0.350	0.014
Tissue and phosphate solution.....	0.006	0.010	0.004	0.008	0.028	0.020	0.0084	0.0137	0.005

Experiments with liver tissue and alanine.

III. Three weeks' incubation:									
Tissue and alanine.....	0.003	0.012	0.008	0.310	0.362	0.052	0.346	0.455	0.109
Tissue and phosphate solution.....	0.005	0.011	0.006	0.026	0.068	0.042	0.045	0.178	0.133
IV. One-week incubation:									
Tissue and alanine.....	0.006	0.010	0.004	0.322	0.330	0.008	0.370	0.434	0.064
Tissue and phosphate solution.....	0.006	0.013	0.007	0.012	0.037	0.015	0.053	0.128	0.075

*Experiments with leucocytes.**Without antiseptics.*

V. Three weeks' incubation:									
Leucocytes and alanine.....	0	0.014	0.014	0.332	0.398	0.066	0.384	0.520	0.136
Leucocytes and phosphate solution.....	0	0.007	0.007	0.004	0.073	0.067	0.056	0.192	0.136

NOTE ON A CASE OF PENTOSURIA.

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The presence of pentose in urine was first demonstrated by Salkowski¹ in 1892. Later Neuberg² undertook an investigation into the nature of the pentose. He came to the conclusion that the pentose was *dl*-arabinose. Numerous observers corroborated Neuberg's statement, inasmuch as they found that urine showing the presence of pentose, as a rule, was optically inactive. Only a few instances are recorded in which the urine containing pentose showed optical activity. In the few cases recorded by Luzzatto,³ Blumenthal,⁴ and by Schüler,⁵ the urines were dextro-rotatory. Blumenthal and Schüler have taken for granted that the sugar present in the urine was arabinose and hence regarded the pentose observed by them as *l*-arabinose. Luzzatto prepared the phenyl osazone of the pentose present in the urine of his patient and observed a rotation of $+1.04^{\circ}$ in a concentration which required according to the theory a rotation of $+1.10^{\circ}$. On the basis of this he came to the conclusion that the sugar was *l*-arabinose.

Very recently Elliott and Raper⁶ reported on a case of pentosuria in which the sugar failed to form derivatives with diphenyl hydrazine, nor did they succeed in obtaining any other evidence that the sugar was arabinose, and in fact suggested the possibility that the sugar was ribose.

A few years ago a urine containing a pentose came to our observation. A phenyl osazone prepared from the sugar showed

¹ Salkowski and Jastrowitz: *Centralbl. f. d. med. Wiss.*, xxx, p. 337, 1892.

² Neuberg: *Ber. d. deutsch. chem. Gesellsch.*, xxxiii, p. 2243, 1900.

³ Luzzatto: *Hofmeister's Beiträge*, vi, p. 87, 1905.

⁴ Blumenthal: *Realenzyklopädie*, xxxii, p. 388, 1903.

⁵ Schüler: *Berl. klin. Wochenschr.*, xlvii, p. 1322, 1910.

⁶ Elliott and Raper: *this Journal*, xi, p. 211, 1912.

dextro-rotation of $+0.86^{\circ}$ in a concentration and in a solution prepared according to the directions of Neuberg. Under such conditions *l*-arabinose shows a rotation of $+1.10^{\circ}$, and *l*-xylose a rotation of $+0.15^{\circ}$. Hence it was evident that the pentose present in the urine under our observation belonged to the arabinose group. In fact, very frequently in course of the work on nucleic acid phenyl osazone of *d*-ribose was obtained which in the same concentration and in the same solution as used in the present experiment showed an identical rotation of -0.86° . An attempt was therefore made to establish the nature of the pentose. Great difficulties, however, were encountered in the attempt to obtain the sugar in a higher concentration. The urine contained only 0.25 per cent of the pentose (calculated on the basis of glucose). At first an attempt was made to concentrate the urine at a pressure of about 18 mm. and a temperature of about 40° . However, when the urine was concentrated to a thick syrup, it gave only a slight test with orcin and possessed only insignificant reducing power for Fehling's solution. Evidently the greater part of the pentose was either destroyed or had undergone some condensation so that the usual derivatives of it were no longer obtainable.

It is worthy of note that Luzzatto had already called attention to the fact that the concentrating of the urine of his patient led to a considerable loss of the pentose. It was necessary to find a way by which the sugar could be obtained in greater concentration than present in the urine before any more detailed investigation into the nature of the pentose could be undertaken. It was found that the addition of weak acids or of barium carbonate to the urine to some extent improved the condition so that the pentose could be obtained in concentration of 1 to 2 per cent. But, even then, the concentrated urine showed the evidence of considerable decomposition of the pentose.

An attempt was then made, first to remove the urea of the urine and then to obtain the pentose by means of a solution of lead acetate and of barium hydrate. The lead precipitate of the pentose was then decomposed in the usual way and the aqueous solution containing the sugar concentrated under diminished pressure and at a low temperature. Solutions obtained in that manner contained very little nitrogen but at the end of concentration

again showed a considerable destruction of the pentose. Also this method, therefore, was abandoned. Finally it was concluded to dry the lead precipitate of the sugar and then to extract it with alcohol containing just sufficient hydrochloric acid to remove the lead. In this manner an alcoholic solution of the pentose was obtained. It was hoped that this alcoholic solution of the pentose could be used directly for the preparation of the various hydrazones. However, in no instance was it possible to obtain a diphenyl hydrazone characteristic for arabinose. Even in solutions which contained the pentose in a fairly high concentration an insoluble hydrazone could not be obtained. On the other hand, the addition of arabinose either directly to the urine, or to the concentrated urine, or to the solutions obtained on decomposition of the lead derivatives always led to the formation of the hydrazone. The failure to obtain the diphenyl hydrazone naturally can be interpreted in two ways: first, that the urine contained some substances which prevented the hydrazone from crystallizing; and second, that the pentose was not arabinose, but either ribose or keto-arabinose. It is worthy of note that the residue obtained on evaporation of the alcoholic extract from the lead compound of the sugar contained a large proportion of organic non-nitrogenous material which was not the pentose. It is undoubtedly due to the presence of these substances that a crystalline hydrazone of the pentose was not obtainable. It was also impossible to obtain in crystalline form an oxidation product of the pentose.

In order to obtain some information into the nature of the pentose the optical rotation of the sugar solution was compared with its reducing power for Fehling's solution. In this manner it was found that the specific rotation of the sugar was $+17.5^\circ$. The specific rotation of *l*-arabinose is $+104.4^\circ$ and that of ribose is $+19.3^\circ$. On the basis of this it seems more probable that the pentose present in the urine under our observation is not *l*-arabinose but *l*-ribose. We fully realize, however, that the exact nature of the sugar cannot be made certain before it is obtained in a purity that will permit the obtaining of some well-defined derivatives.

This report is made at this stage for the reasons, that on the one hand the material is not accessible for further work, and second

that it suggests the possibility that there may exist more than one form of pentosuria.

EXPERIMENTAL.

Osazone.

400 cc. pentose urine were heated on the water bath with 5 grams of phenyl hydrazine. The osazone began to separate almost at once and after one hour the crystals were filtered off and recrystallized from water containing pyridine. It melted at 163°C.

0.1264 gram substance gave 0.0712 gram H₂O and 0.2872 gram CO₂.

	Calculated for C ₁₇ H ₂₀ O ₄ N ₄ :	Found:
C.....	62.19	61.98
H.....	6.10	6.24

0.1002 gram osazone in 5 cc. pyridine alcohol rotated in a 0.5 dm. tube with D-light + 0.43°.

5 liters of urine were precipitated with mercuric acetate and sodium hydrate. The mercury was removed from the filtrate with hydrogen sulphide and a small amount of lead acetate solution added to remove the excess of the latter. The filtrate was then treated alternately with a solution of basic lead acetate and barium hydrate until no further precipitate was produced. The lead and barium precipitate was washed with water and decomposed by shaking with 5 per cent sulphuric acid. The excess of the sulphuric acid was removed with barium carbonate and the amount of sugar calculated as glucose found by titration to be 3 grams. The solution could not be concentrated without destruction of the sugar.

2 cc. of solution corresponded to 13.5 cc. ammonium sulphocyanate (1 cc. $\frac{N}{10}$ ammonium sulphocyanate = 377 mgm. ribose).⁷ Hence, 100 cc. contained 2.54 grams calculated from the absolute reducing power of ribose. It rotated in a 2 dm. tube with D-light + 0.91°.

$$[\alpha]_D^{20} = +17.5^\circ$$

1 PER CENT RIBOSE SOLUTION	FEHLING'S SOLUTION	$\frac{N}{10}$ SULPHOCYANATE	FOR 1 CC.
cc.	cc.	cc.	
2	14	4.70	2.35
3	21	8.30	2.76
4	28	10.40	2.60
4	28	10.60	2.65
5	35	13.30	2.66
7	49	18.50	2.60

⁷ The exact data regarding the reducing power of ribose for Fehling's solution are as yet lacking. The following experiments were made prin-

Concentration of the pentose.

By extracting the dried precipitate from the lead and barium with warm alcoholic hydrochloric acid a solution of the pentose was obtained, which after removal of the hydrochloric acid with an excess of lead carbonate could be concentrated to a syrup which is free from nitrogen. However, the total weight of the residue exceeded enormously the calculated weight of sugar in it.

Attempts to prepare hydrazones of the pentose.

20 cc. of a solution containing 0.4 gram sugar were treated with 0.5 gram parabromophenyl hydrazine and the solution concentrated in vacuum at room temperature in a desiccator. A syrup was left which did not crystallize.

The experiment was performed with benzyl phenyl hydrazine with addition of an equal volume of alcohol and boiling for one hour on the water bath. Upon concentrating in vacuum only a syrup was obtained which did not crystallize.

The same experiment with diphenyl hydrazine also led to a negative result. The last two experiments were repeated. The addition of 0.4 gram of arabinose yielded the respective hydrazones.

5.6 grams sugar in 750 cc. of water were treated with 3.9 grams (1 mol.) phenyl hydrazine and the solution evaporated at low temperature in vacuum. Instead of a hydrazone the above described osazone was obtained.

cipally to obtain the factor required for the present work. More detailed and numerous determinations are still needed. The reducing power of ribose was first determined approximately by varying the amount of Fehling's solution required to reduce a given amount of 1 per cent solution, and the following determinations carried out by Volhard's method, using the required volume of the same concentration of Fehling's solution.

A NEW METHOD FOR THE DETERMINATION OF UREA IN BLOOD.

By E. K. MARSHALL, JR.

(From the Laboratory of Physiological Chemistry, Johns Hopkins University.)

(Received for publication, July 14, 1913.)

In a recent number of this *Journal*,¹ a new method for the estimation of urea in urine was described. This method consists in the conversion of the urea into ammonium carbonate by means of the urease of the soy bean, and an estimation of the alkalinity before and after the conversion by means of standard acid and methyl orange. The procedure is not directly applicable to the estimation of urea in blood serum owing to the large quantity of proteins and small amount of urea present. However, by a conversion of the urea into ammonium carbonate with the use of the enzyme, and a subsequent removal of the ammonia by means of an air current as suggested by Folin,² satisfactory results can be obtained without a preliminary removal of the proteins, as is necessary in other methods for the determination of urea in blood.

Procedure. The blood is drawn in the usual manner and allowed to stand on ice until clotting is complete. As shown below the urea content of the serum does not change after standing even for three or four days; the blood can, therefore, be kept on ice over night, if desired.

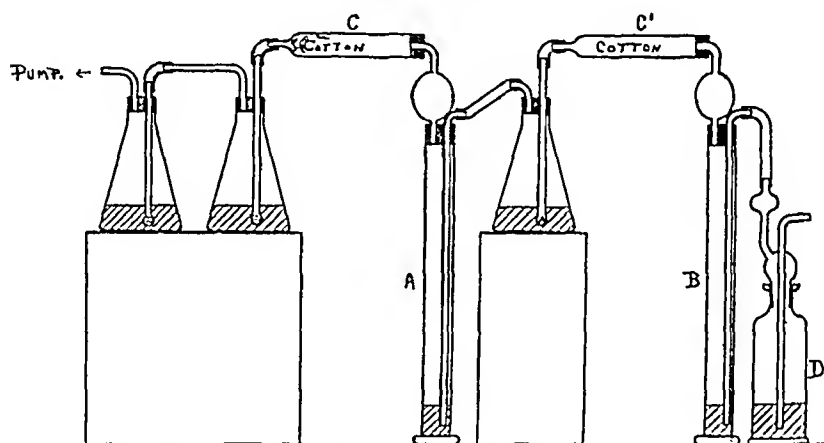
Two equal portions of the serum are measured into ordinary test tubes, 1 cc. of the soy bean extract³ added to one tube, and

¹ This *Journal*, xiv, p. 283, 1913.

² *Zeitschr. f. physiol. Chem.*, xxxvii, p. 161, 1902-03.

³ The preparation of the soy bean extract is described in the previous article. Ten grams of finely ground soy beans are treated with 100 cc. of water and allowed to stand with occasional agitation for one hour. 10 cc. of $\frac{N}{10}$ hydrochloric acid are added and the mixture allowed to stand about fifteen minutes longer. It is now filtered and preserved with toluene. Such a solution is perfectly satisfactory for use at least five or six days after its preparation.

about 0.5–1.0 cc. of toluene to each. If sufficient serum is available, 10-cc. portions should be used; however, perfectly satisfactory results can be obtained by using 5-cc. or even 3-cc. portions of the serum. The tubes are tightly stoppered, and allowed to remain at room temperature until the conversion of the urea into ammonium carbonate is complete. Generally, they are allowed to stand over night, although four to five hours is usually amply sufficient for the completion of the reaction. The contents of the tube containing the serum and extract are transferred to cylinder A, and washed in with a very small amount of water (not more



than 5 cc.). Two grams of sodium chloride, an equal volume of alcohol and a layer of kerosene oil are added to the cylinder. The contents of the other tube are transferred to cylinder B, and treated in exactly the same manner. 25 cc. of $\frac{N}{10}$ hydrochloric acid and about 25 cc. of water are placed in each of the 200 cc. Erlenmeyer flasks used for the absorption of the ammonia. The different parts of the apparatus are now connected and about 0.5 gram of sodium carbonate added to each cylinder. A rapid air current is passed through the apparatus until all the ammonia has been removed from the cylinders. With a good suction pump, one hour suffices. The excess of acid in the absorption flasks is titrated with $\frac{N}{10}$ sodium hydroxide and alizarin sodium sulphate. The amount of acid neutralized in the flask attached to cylinder

B corresponds, of course, to the ammonia⁴ present in the serum, while the amount used in the other two flasks represents the urea plus the ammonia. The difference corresponds to the urea in terms of $\frac{N}{37}$ hydrochloric acid, and multiplied by 0.0006 gives the urea in grams present in the amount of serum taken for the determination.

Details in connection with the apparatus and determination. 1. On account of the large quantity of protein in serum, it was found advisable to use both alcohol and kerosene to prevent foaming.⁵

2. The tubes *C* and *C'* are ordinary calcium chloride drying tubes packed loosely with cotton. These in conjunction with the bulbs prevent any splashing or mechanical transmission of the alkali into the absorption flasks. While the bulbs are probably not absolutely necessary, they are convenient in keeping the cotton filters dry.

3. For the better absorption of the ammonia, the tubes in the Erlenmeyer flasks are closed at one end, and pierced with six or seven small holes, as suggested by Folin.⁶ Even with this device one absorption flask is not always sufficient to completely absorb the ammonia. Two flasks are always used for safety in connection with the urea determination; however, since from the serum alone only a very small amount of ammonia (corresponding to 0.10–0.70 cc. of $\frac{N}{37}$ HCl) is ordinarily obtained, one absorption flask is here sufficient.

4. A layer of toluene is placed on the liquid in the absorption flasks, for, due probably to the alcohol carried over by the air current, considerable foaming sometimes occurs. If not prevented, this results in a loss of a portion of the contents of the flask.

5. The bottle *D* contains dilute sulphuric acid to free the air from any traces of ammonia before passing it through the apparatus.

⁴ We can, however, place no value on this as a determination of the true ammonia content of the blood; for on standing even a few hours the blood develops much more ammonia than the original amount (Folin: *this Journal*, xi, p. 527, 1913).

⁵ This has been earlier pointed out by Folin in connection with the use of the air-current method for determining ammonia in blood (*Zeitschr. f. physiol. Chem.*, xxxvii, p. 165, 1902–03).

⁶ *This Journal*, xi, p. 493, 1912.

6. No correction is necessary for the ammonia derived from the 1 cc. of soy bean extract used, as the amount obtained from this source is inappreciable.

The following table shows the results obtained with various preparations of the extract.

AGE OF EXTRACT	AMOUNT TAKEN	$\frac{N}{50}$ HCl REQUIRED	$\frac{N}{50}$ HCl CAL. FOR 1 CC.
	cc.	cc.	cc.
fresh	2	0.04	0.02
fresh	10	0.20	0.02
18 hrs.	5	0.28	0.06
3 days	10	0.33	0.03
5 days	4	0.13	0.03

Determination of pure urea solutions. The following table presents the results obtained by the application of this method to very small amounts of urea, and also to mixtures of urea and ammonium sulphate.

GRAMS UREA TAKEN	GRAMS UREA FOUND	GRAMS UREA TAKEN	GRAMS UREA FOUND
0.00066	0.00069	0.01100	0.01080
0.00066	0.00072	0.01120	0.01110
0.00110	0.00114	0.01120	0.01193
0.00110	0.00108	0.01263	0.01278
0.00330	0.00310	0.01263	0.01243
0.00506	0.00507	0.02526	0.02526
0.00550	0.00550	0.02526	0.02523

GRAMS UREA TAKEN	GRAMS $(\text{NH}_4)_2\text{SO}_4$ TAKEN	GRAMS UREA FOUND	GRAMS $(\text{NH}_4)_2\text{SO}_4$ FOUND
0.00132	0.00079	0.00138	0.00084
0.00220	0.00132	0.00217	0.00134

Determination of urea added to blood. Varying small amounts of urea were added to blood serum and the amount of urea estimated before and after the addition. Such a procedure demonstrates that the method is capable of yielding all the urea present in the serum; however, it does not prove that other substances may not be undergoing decomposition at the same time. Tacheuchi

found that the urease of the soy bean was not capable of yielding any ammonia from guanidine, arginine, benzamide, allantoin, leucine, alanine, tyrosine, creatine, histidine, guanine, glycocholic acid or hippuric acid.⁷ Hence it appears very probable that no other constituents of the blood (or urine) contribute ammonia to the final result when urea is determined by this method.

SOURCE OF BLOOD	UREA: GRAMS IN 5 CC. SERUM	UREA ADDED TO 5 CC. SERUM	UREA FOUND	DIFFERENCE: ADDITIONAL UREA
Pig.....	0.00066	0.00510	0.00566	0.00500
	0.00071	0.00517	0.00585	0.00514
	0.00071	0.00517	0.00589	0.00518
	0.00069	0.00517	0.00573	0.00504
	0.00069	0.00517	0.00582	0.00513
	0.00150	0.01000	0.01092	0.00942
	0.00072	0.01000	0.01074	0.01002
Dog.....	0.00157	0.00505	0.00654	0.00497
	0.00157	0.01263	0.01395	0.01238
	0.00138	0.01263	0.01318	0.01180
	0.00110	0.00505	0.00621	0.00511
	0.00070	0.00208	0.00267	0.00197

Duplicate determinations by this method agree quite closely. On a sample of human blood was obtained 0.477 and 0.483 gram per liter; on a sample of pig's blood, 0.144, 0.144, 0.141, 0.147 gram per liter; and on a sample of dog's blood, 0.276, 0.267, 0.270 gram per liter. A considerable number of determinations have been made by this method on human blood under pathological conditions and will be reported elsewhere.

Stability of urea in serum. Since in the method outlined above, the serum is allowed to stand over night, it is of great importance to determine whether the urea content of the serum is diminished by this procedure. The figures cited below prove that serum allowed to remain on ice for even four days does not appreciably change in its urea content. The serum was allowed to remain in an ice chest, portions pipetted off at various intervals and the urea estimated. The figures represent grams of urea per liter of serum.

⁷ *Journal of the College of Agriculture, Tokyo*, i, p. 1, 1909.

Pig's serum: April 22, 0.144; April 23, 0.141; April 24, 0.138; April 26, 0.147.

Dog's serum: (a) April 25, 0.174; April 28, 0.177 (b) May 10, 0.279; May 12, 0.276.

Also, the stability of urea in serum is shown by the fact that if urea is added to serum and its value determined immediately, and then the serum allowed to stand over night at room temperature and the urea determined, practically the same figures are obtained. In the first instance, in the following table, the urea was determined at once by adding 1 cc. of the extract and allowing the serum to stand for four hours; in the other two cases the serum was allowed to remain over night before estimating the urea.

GRAMS UREA IN 5 CC. SERUM	GRAMS UREA ADDED TO 5 CC.	GRAMS UREA FOUND	DIFFERENCE
0.000705	0.00517	0.00571	0.00501
0.000705	0.00517	0.00585	0.00515
0.000705	0.00517	0.00588	0.00518

However the amount of acid required for the blank increases, that is, the ammonia present in the serum is increased. The increase in ammonia does not come from the urea, but from the proteins or some other source.

Comparison of this method with Folin's method. A few comparative figures of the results obtained by this method with those obtained by Folin's new method³ for determining urea in blood, are cited below. Dog's blood serum was used in each instance. Since the blood was allowed to clot, and stand for four or five hours, its ammonia content was, of course, increased. In Folin's method the blood is transferred immediately to methyl alcohol as soon as it is drawn, consequently no correction for the ammonia present is necessary. Here, however, we would have a correction for the ammonia, but since the ammonia determinations were not made at the same time as the serum was transferred to methyl alcohol, we have preferred to give the uncorrected results. This explains in some measure the higher results obtained by

³ This *Journal*, xi, p. 527, 1912.

Folin's method. All results are calculated in grams of urea per liter of serum.

Urease method.	0.372	0.360	0.180	0.324	0.278	0.291	0.288
Folin's method.	0.408	0.396	0.234	0.321	0.310	0.338	0.300

Application of the method to body fluids and tissues. An application of the method described in this paper to the more exact determination of urea in urine than is afforded by the rapid clinical method previously described is shown in a following paper. The method should lend itself readily to the estimation of urea in other body fluids and tissues. In fluids, the urea can generally be determined without preliminary treatment; while tissues must be first extracted with alcohol, the alcohol extract evaporated, and the residue dissolved in water before applying the method. The question of inhibitory substances must, of course, be considered in this connection. If a liquid contains acids or mercuric chloride in appreciable amount, the presence of these will completely arrest the action of the enzyme. The following procedure is useful in testing a fluid for inhibitory substances. A quantity of the fluid, equal in amount to that taken for the determination is measured into a test tube, a couple of drops of phenolphthalein solution added, and a few crystals of pure urea. 1 to 2 cc. of the soy bean extract are added and provided no inhibitory substances are present, the red color of the indicator should appear in about five to ten minutes. Armstrong has recently⁹ studied the action of certain substances on urease. Of the substances studied, quinone, quinol and formaldehyde had an extreme inhibitory effect, while amino-acids and other weak acids in small amount accelerated the action slightly.

A few results showing the application of the method to the determination of urea in sweat are cited below.

UREA FOUND IN 5 CC. SWEAT	UREA ADDED TO 5 CC. SWEAT	UREA FOUND	DIFFERENCE
0.0140	0.00416	0.01812	0.00412
0.00825	0.00552	0.01359	0.00534

⁹ Armstrong, Benjamin and Horton: *Proc. Roy. Soc., B*, lxxxvi, p. 328, 1913.

A more extensive investigation of the action of acids and alkalis on this enzyme, as well as its behavior in the presence of various substances, will be investigated to determine the value and limitations of the method in estimating urea in miscellaneous fluids.

THE DETERMINATION OF UREA IN URINE.

(SECOND COMMUNICATION.)

By E. K. MARSHALL, JR.

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The method outlined in the foregoing paper for the estimation of urea in blood is perfectly reliable for larger quantities of urea (up to 25 mgms. or perhaps more). Its adaptation to the determination of urea in urine using only a small amount of the urine is shown in this paper. The method to be described is slightly more accurate than the rapid clinical method proposed earlier.¹ The reason for this is twofold; the rapid titration method gives results slightly lower than the theoretical on account of the presence of carbon dioxide during the titration, and titrations performed in a mixture such as urine are not as accurate as in the case of the neutralization of a pure solution of an acid by an alkali.

Two 1-cc. portions of the urine are measured into test tubes by means of an Ostwald pipette.² The urine is now diluted to about 10 cc., 1 cc. of the soy bean extract added to one tube and from 0.5–1 cc. of toluene to each. The tubes are allowed to remain at room temperature over night. With the following exceptions, the remainder of the procedure is exactly similar to that described in the foregoing article on the determination of the urea in blood. No alcohol need be added to the contents of the cylinders, for oil alone suffices to prevent excessive frothing. The first absorption flask attached to the cylinder containing the urine and the ferment extract is charged with 50 cc. of $\frac{N}{30}$ hydrochloric acid instead of 25 cc. In the case of pathological urines

¹ This *Journal*, xiv, p. 483, 1913.

² These pipettes are the same as those used by Folin (this *Journal*, xi, p. 493, 1912) and can be obtained from Eimer and Amend. However, should 5 cc. of the urine be available, it is equally convenient to dilute this to 50 cc. and use two 10-cc. portions for the determination.

containing large amounts of ammonia two absorption flasks may be necessary for each cylinder.³

Instead of the foregoing procedure in which the urea and ammonia are determined in one operation, the ammonia can be obtained by the air-current method in the usual manner using larger amounts of urine. Then, only one portion of the apparatus is necessary for the urea estimation.

A comparison of the results obtained by this method, the rapid clinical method previously described, and the new phosphoric acid method⁴ proposed by Folin is given in the table below. Folin⁵ has recently compared the phosphoric acid method with his magnesium chloride method and Benedict⁶ method, and found that the three methods yield practically identical results. In using the phosphoric acid method, two absorption flasks were found necessary instead of one. The results are expressed in grams of urea per liter of urine.

RAPID METHOD	NEW METHOD	FOLIN'S PHOSPHORIC ACID METHOD
19.50	19.80	19.86
11.80	12.00	11.60
7.98	8.04	7.95
15.12	15.12	15.18
25.46	25.08	25.08
11.10	11.37	11.10
19.62	19.80	19.68
	15.09	15.00
19.65	20.19	20.25
21.42	22.02	21.66
9.06	9.15	9.12
19.20	19.38	19.00
19.50	20.22	20.28

Hence, it appears from the above table that the rapid method gives results which are perfectly reliable, while the results obtained by the method here described and Folin's phosphoric acid method are practically the same.

³ Of course the special ammonia-absorption tubes devised by Folin might be used and hence only one absorption cylinder would be necessary in each case. However, with the very dilute solutions used for titration, it appeared better to keep the volume as small as possible.

⁴ This *Journal*, xi, p. 512, 1912.

⁵ *Ibid.*, xi, p. 511, 1912.

⁶ *Ibid.*, viii, p. 405, 1910.

BLOOD GLYCOLYSIS: ITS EXTENT AND SIGNIFICANCE IN CARBOHYDRATE METABOLISM. THE SUPPOSED EXISTENCE OF "SUCRE VIRTUEL" IN FRESHLY DRAWN BLOOD.

BY J. J. R. MACLEOD.

(WITH THE COLLABORATION OF A. M. WEDD.)

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(Received for publication, July 24, 1913.)

After a lapse of several years during which it was largely neglected, the subject of glycolysis has recently received considerable attention and with important and far-reaching results.¹ These researches have dealt with both tissue and blood glycolysis. In connection with the former although much has been added to our knowledge of the rate of sugar consumption by the surviving heart it has, so far, been impossible to demonstrate that true glycolytic power exists in the case of isolated animal tissues or juices.²

The greater part of the work within the past year or so has been concerned with blood glycolysis and it may be of interest to review some of the results that have been obtained. In the first place it seems clear that the activity of the glycolytic process varies extremely in the blood of different classes of animals. Thus A. Loeb³ has found that glycolysis is most marked in the blood of the dog, being also considerable in that of man and sheep; on the other hand it has been found almost absent in the blood of the ox and pig.⁴

¹ For full literature concerning the older researches consult Oppenheimer: *Die Fermente*, 2er Aufl. (Vogel, Leipzig, 1910); for more recent work, von Fürth: *Probleme der physiologischen und pathologischen Chemie*, Bd. ii (Vogel, Leipzig, 1913).

² Levene and Meyer: *This Journal*, xi, pp. 347-353, 1912.

³ Loeb, A.: *Biochemische Zeitschr.*, xlix, p. 413, 1913.

⁴ Cf. also Melvin, G. S.: *Biochemical Journ.*, vi, p. 422, 1912. Melvin could detect no glycolysis either in ox- or sheep-blood.

It has been pointed out by this observer that the bloods which exhibit feeble glycolytic power are those in which normally the red corpuscles contain only a small percentage of dextrose, it having been previously established by Masing⁵ that the distribution of dextrose between corpuscles and plasma varies very considerably in the blood of different classes of animals. After the addition of dextrose to blood, the corpuscles of which normally contain little dextrose (and which therefore possess little glycolytic power) no evidence of penetration of the dextrose into the corpuscles could be obtained by Masing. Taking all the facts together, Loeb has formulated the hypothesis that the glycolytic process resides within the red corpuscles, through the envelope of which the sugar must therefore pass before it can be destroyed. Two factors might therefore influence the rate of glycolysis in whole blood: the diffusibility of the corpuscular envelope towards sugar and the glycolytic power of the corpuscular contents. By observations on the relative disappearance of dextrose from serum and corpuscles respectively, Loeb has furnished very strong evidence in favor of his hypothesis, having found that the amount of sugar in the corpuscles shows only a slight decrease, but that in the serum the decrease is marked, in those bloods (dog) in which the envelope is very permeable towards this substance. While not denying that erythrocytes may possess glycolytic powers, Levene and Meyer have shown that leucocytes are also endowed with this property and Rona and Arnheim⁶ have found that both leucocytes and erythrocytes of "oxalate" blood of the dog and cat can destroy dextrose in the presence of phosphates. It was impossible in these observations to determine in which variety of corpuscle this power was the more marked.

Among other important recently established facts regarding blood glycolysis may be cited the following:

1. Serum has no glycolytic power (Rona and Döblin,⁷ von Noörden, Jr.⁸).

2. The glycolytic power of the blood of different individuals of the same species may vary considerably. For example, in the

⁵ Masing: *Pflüger's Archiv für die gesamte Physiologie*, cxlix, p. 227, 1912.

⁶ Rona and Arnheim: *Biochem. Zeitschr.*, xlviii, p. 35, 1913.

⁷ Rona and Döblin: *ibid.*, xxxii, p. 489, 1911.

⁸ von Noörden, Jr.: *ibid.*, xlv, p. 94, 1912.

case of the blood of the dog the following values, expressing the disappearance of dextrose as a percentage of the amount of dextrose originally present (percentile glycolysis) have been found.

After 5 hours at 37°C., between 25 and 26.

After 6 hours at 37°C., between 20.6 and 41.6 (Rona and Arnheim).

After 1½ hours at 40°C., between 48.5 and 62.7 (A. Loeb).

After 2 hours at 37°C. (2 per cent oxalate), between 15 and 18.6.

After 6 hours at 37°C., between 59.7 and 88.4 (Edelmann).⁹

After 90 minutes at 40°C., between 33 and 58.

After 2 hours at 40°C., between 30 and 32 (Kondo).¹⁰

3. Although the glycolytic process has been said to be depressed by the addition of fluoride to the blood, it is claimed by certain authors that oxalates in sufficient amount to prevent clotting do not have this effect. Rona and Arnheim¹¹ state that 0.1 per cent oxalate causes no depression. Edelmann employed blood containing 0.2 per cent oxalate, but, as a general rule, the glycolysis in his observations was slower than that observed by other workers.

4. Dilution of blood with water destroys the glycolytic power, which is not the case when the dilution is made with Ringer's solution or with a phosphate mixture. Besides the H ion concentration, the phosphate and carbonate ions seem to be important for the process. Mere laking of the blood, as by means of saponin, does not destroy the glycolytic power.¹²

5. Although oxygen favors glycolysis to a certain extent, it can nevertheless proceed anaerobically (Rona and Döblin). The glycolytic power disappears after the blood has stood for some time outside the body.

6. The addition of dextrose to blood does not increase the absolute amount of glycolysis occurring in a given time; it therefore lowers the percentile glycolysis (Rona and Döblin).¹³

In the present research several of the conclusions mentioned in the above review have been put to the test and observations have been made with the object of demonstrating whether the process of blood glycolysis plays any rôle in the consumption of carbohydrate in the intact animal.

⁹ Edelmann: *Biochem. Zeitschr.*, xl, p. 314, 1912.

¹⁰ Kondo: *ibid.*, xlv, p. 88, 1912.

¹¹ Rona and Arnheim: *loc. cit.*

¹² Edelmann: *loc. cit.*

¹³ Rona and Döblin: *loc. cit.*

EXPERIMENTAL.

Methods. The blood from the femoral vein or artery of etherized dogs was collected in sterile flasks, under strict aseptic precautions, and the animal was, as a rule, allowed to recover from the operation. In most of the experiments the blood was defibrinated by means of glass beads and quantities of 15 cc. each were transferred to small sterile Erlenmeyer flasks which, after being closed by sterile cotton plugs and tin foil, were suitably fixed to a holder which was kept in motion by means of a motor. The holder was placed in a water bath at 40°C. After varying periods of time the flasks were removed and the sugar was determined in the incubated blood by Bertrand's method, the proteins having been first of all removed by means of colloidal iron. The sugar was similarly determined in a control sample of blood before incubation. Although in the few hours during which the blood was incubated in our experiments, there could have been little danger of bacterial destruction of sugar, yet in all the earlier observations careful bacteriological examinations were made, but with negative results. For those examinations we are indebted to Mr. W. W. Donaldson.

Glycolysis in oxalate and in defibrinated blood.

It was found that the method employed for preserving the blood in a fluid condition has an influence on the rate of glycolysis in that oxalate, beyond a concentration of one per thousand, distinctly retards it. This is shown in the following table in

TABLE I.

NO. OF EXP.	TIME OF INCUBA- TION	OXALATE BLOOD				DEFIBRINATED BLOOD	
		Per cent dextrose in blood at start	Per cent oxalate	Dextrose dis- appeared from 100 gms. blood	Percentile glycolysis	Dextrose dis- appeared from 100 gms. blood	Percentile glycolysis
	hours			grams		grams	
	3	0.126	0.1	0.017			
	5			0.034	27	0.079	66.8
	4½		0.34	0.033	16.6	0.085	63

which are given the amounts of dextrose disappearing from 100 grams of blood and the *percentile glycolysis*¹⁴ for blood that was either received into a sterile solution of potassium oxalate or was defibrinated in a sterile flask by shaking with glass beads.

The following table shows that the depression of glycolytic power is more or less proportional to the concentration of oxalate.

TABLE II.

NO. OF EXP.	TIME OF INCUBATION	PER CENT DEXTROSE IN BLOOD AT START	PER CENT OXALATE	DEXTROSE DISAPPEARED FROM 100 GMS. BLOOD	PERCENTILE GLYCOLYSIS
	<i>hours</i>			<i>grams</i>	
2	2	0.195	0.128	0.011	5.6
	5½			0.092	47.2
2	2	0.200	0.178	0.008	4.8
	5½			0.068	33.8
1	2	0.216	0.4	0.004	
	3¼			0.021	9.2

The depressing influence of the oxalate is not dependent on the fact that clotting has been prevented but is due to an influence of the oxalate itself. This was demonstrated by studying the effect produced on glycolysis by adding various amounts of oxalate to defibrinated blood and by comparing the glycolysis in unclotted "hirudin" blood with that occurring in another sample of the same blood after defibrination. Table III gives the results of these experiments.

Although we cannot say from the above experiments whether it would be possible to add a sufficiency of oxalate to prevent clotting without interfering with glycolysis, yet it is evident that with the amount of this substance usually employed for this purpose (viz., 0.1 per cent) considerable interference occurs. If unclotted blood is required, as when the glycolytic powers of corpuscles and plasma are to be separately investigated, hirudin should therefore be employed.

The same influence of oxalate on glycolysis was observed in blood which had been laked by means of saponin. Thus in laked blood containing about 0.5 per cent oxalate only 0.004 gram of dextrose disappeared in three hours, whereas without oxalate

¹⁴ Percentile glycolysis means the decrease of dextrose per 100 grams of blood as a percentage of the amount of dextrose originally present.

0.0405 gram of dextrose disappeared, the original percentage of dextrose in both cases being 0.096.

TABLE III.

NO. OF EXP.	TIME OF INCUBATION	PER CENT DEXTROSE IN BLOOD AT START	DEFIBRINATED BLOOD		DEFIBRINATED BLOOD + OXALATE OR HIRUDIN		
			Dextrose disappeared from 100 gms. blood	Percentile Glycolysis	Per cent oxalate or hirudin	Dextrose disappeared from 100 gms. blood	Percentile Glycolysis
	<i>hours</i>		<i>grams</i>			<i>grams</i>	
5	3	0.101	all?	100?	oxalate	0.042	41.7
7	2½	0.154	0.084	54.2	0.125 oxalate	0.053	34.4
8	3	0.079	0.038	48.2	indefinite hirudin	0.023	29.8
	2¾		0.044	55.6	1 mg. to 8 gms.	0.056	71.8
	4½		0.064	81.2		0.064	81.2
9	½	0.162	0.021	12.9	hirudin, 1 mg. to 8 gms.	0.021	12.9
	2½		0.077	47.5		0.081	50

The average rate of glycolysis in defibrinated blood at 40°C.

Although a great part of the discordance between the results of previous observers as to the intensity of glycolysis is to be explained by differences in the method used for keeping the blood fluid, and in the source of the blood, yet, even in the case of defibrinated dog-blood, glycolysis does not always proceed at the same rate. To study the variations we have compared: (1) the glycolysis occurring in blood collected at different periods from the same dog living meanwhile under practically constant conditions and (2) that occurring in the blood of different dogs. The values given in the following table were found in the blood of four dogs observed at different periods:

TABLE IV.

EXP. NO.	DATE	TIME OF INCUBATION	PER CENT DEXTROSE IN BLOOD AT START	DEXTROSE DISAPPEARED FROM 100 GMS. BLOOD	PERCENTILE GLYCOLYSIS
		<i>hours</i>		<i>grams</i>	
A 3	III, 3	5	0.119	0.079	66.8
15	III, 27	3	0.134	0.083	
		5½		0.103	76.5
B 2	III, 4	2¼	0.129	0.059	45.4
22	IV, 21	2¼	0.106	0.047	44.2
C 5	III, 11	3	0.101	all?	
17	IV, 7	3	0.104	0.071	
D 8	III, 17	2¼	0.079	0.044	55.6
		4½		0.064	81.1
12	III, 21	3	0.205*	0.086	42.4
		5		0.130	63.4

* Hyperglycaemia.

It is seen that distinct variations occur when we consider the absolute amounts of dextrose that disappear during each period. More constant values are however obtained when the decrease is calculated as a percentage of the amount of dextrose originally present (percentile glycolysis). Although such a calculation is probably permissible when the percentage of dextrose is at or about the normal, this is not the case when the percentage is excessively high. We shall give evidence for this statement later (see page 508), meanwhile it is important to note that the discrepancies in dog D are probably to be explained in this way.

Turning now to the observations on different animals, Table V contains figures expressing the percentile glycolysis occurring at different periods in cases where the original percentage of dextrose in the blood was approximately normal.

From such results we cannot form any very accurate estimate of the average velocity of the glycolytic process although in general it is probably safe to conclude that approximately one-quarter of the sugar has disappeared in about one and one-half and about one-half of it in about two and one-half hours.

We have not a sufficient number of observations on any one sample of blood, from which it is possible to construct the velocity

curve of the glycolysis but it is perfectly clear, especially from such results as are given in Table V, that the process is much quicker during the first hour or so than later. It probably proceeds very slowly during the later stages: In one experiment (No. 21) -for example, blood incubated for eighteen hours still contained 0.043 per cent of dextrose, the original percentage being however very high, 0.230. In another case (No. 26), in which the original percentage was normal all of the sugar had disappeared from the blood after twenty-four hours' incubation.

TABLE V.

TIME	PERCENTILE GLYCOLYSIS	NOS. OF EXPERIMENTS FROM WHICH FIGURES WERE TAKEN
30 min.....	12.9	9
40 min.....	14.2	16
1½ hrs.....	27.7	12
2 hrs.....	28.4	16
2¼ hrs.....	44.3; 54.2	20; 7
2½ hrs.....	47.5; 50	9
3 hrs.....	42	12
4 hrs.....	64.5; 59	13; 16
4½ hrs.....	63.1; 81	4; 8
5 hrs.....	66.8; 79; 80.2	3; 17; 25
5½ hrs.....	76	15

The blood constituent responsible for the glycolysis.

In confirmation of the observations of others, we have found that no trace of sugar disappears from serum, at least after incubation for four hours. The agent responsible for glycolysis therefore resides in the corpuscles. In the deposit of centrifuged blood it was found that glycolysis is very rapid, all traces of sugar having disappeared from this in one experiment after ninety minutes' incubation. It was further found however that repeated washing of the corpuscular sediment with isotonic saline solution decidedly depresses the glycolytic power. Thus in two experiments, centrifuged corpuscles that had been repeatedly washed with isotonic saline and had meanwhile stood in this over night, were suspended in equal volumes either of isotonic saline or phosphate mixture or serum, sufficient dextrose being added in each

case to bring the percentage to about 0.3. As the following results show, it was found that very slight, if any glycolysis occurred, even although sterile conditions could not be strictly maintained.

Experiment XXIII.

1. 15 cc. washed corpuscles + 15 cc. serum + dextrose.
 Per cent dextrose at start..... 0.324
 Per cent dextrose after 5 hours' incubation 0.305
2. 15 cc. washed corpuscles + 7.5 cc. saline + 7.5 cc. phosphate mixture (2 pts. NaH_2PO_4 + 8 pts. Na_2HPO_4 , Ph7.4) + dextrose.
 Per cent dextrose at start..... 0.330
 Per cent dextrose after 5 hours' incubation 0.334
3. 15 cc. washed corpuscles + 15 cc. saline + dextrose.
 Per cent dextrose at start..... 0.350
 Per cent dextrose after 5 hours' incubation 0.338

Experiment XXVI.

1. 15 cc. washed corpuscles + 15 cc. serum previously incubated with glycogen.
 Per cent dextrose at start..... 0.376
 Per cent dextrose after 4 hours' incubation 0.332
2. 15 cc. washed corpuscles + 15 cc. serum + dextrose.
 Per cent dextrose at start..... 0.474
 Per cent dextrose after 4 hours' incubation 0.430

The absence or low degree of glycolysis in these experiments cannot be ascribed to there having been a deficiency of corpuscles in the suspensions, for in those of Experiment XXVI haemoglobin determinations by the Haldane method gave readings of 100 and 96 per cent. There may of course have been less haemoglobin in the other experiments but we scarcely think so.

The depression of glycolysis must therefore be due either to a deterioration of the glycolytic power as a result of standing or to a change in the permeability of the corpuscular envelope. Regarding the former possibility (that the glycolytic power itself had deteriorated) we have made observations on the glycolysis occurring in uncentrifuged blood that had stood for varying periods of time, either in the incubator or at room temperature. Table VI gives the results.

The glycolysis, as judged from the absolute amounts of dextrose that disappeared, was not distinctly less than in fresh blood in two of the observations in which the blood had stood in the

incubator for about twenty-four hours, but it was entirely absent in another blood that had stood for two days at room temperature. Since the corpuscular sediment in Experiments XXIII and XXVI had stood for less than twenty-four hours, and at the temperature of running water, we are inclined to attribute the loss of glycolytic power, which they exhibited, to the effect of washing.

It has been suggested (see p. 498) that glycolysis is an intracorpuseular process, in other words, that before being destroyed in unlaked blood dextrose must be absorbed by the corpuscles. If this should prove to be the case, we may imagine that by the process of washing, the envelope becomes so altered that no

TABLE VI.

NO. OF EXP.	CONDITION OF BLOOD BEFORE ADDING DEXTROSE	TIME OF INCUBATION	PER CENT OF DEXTROSE IN BLOOD AT START	DEXTROSE DIS- APPEARED FROM 100 GMS. BLOOD
		hours		grams
19	Stood two days; sugar-free	3	0.519	0.001
26	Incubated 24 hrs.; sugar-free	4	a. 0.351 b. 0.655	0.056 0.072
21	Incubated over night; con- tained 0.043 per cent dextrose	4	0.329	0.075

absorption of dextrose can occur, hence no glycolysis. That such washing does alter the permeability of the envelope has been shown by Rona and Michaelis.¹⁵ Laking of the blood does not however interfere with glycolysis, thus, in one experiment (31) the glycolysis in defibrinated blood amounted to 0.06 per cent dextrose in three and one-half hours: in the same blood laked by saponin, 0.073 per cent disappeared. In another experiment (32) 0.0405 per cent dextrose disappeared in three hours from saponin-laked blood.

Without further work it is impossible to say whether glycolysis is brought about by an action of the haemoglobin or of some

¹⁵ Rona and Michaelis: *Biochem. Zeitschr.*, xvi, p. 60, 1909; *ibid.*, xviii, p. 375, 1909.

constituent of the envelope of the corpuscle. The fact that glycolysis can proceed in the absence of free oxygen (see p. 499) would tend to indicate that haemoglobin is not the responsible agent. In confirmation of this we found in one observation that the glycolytic process was not appreciably interfered with by the presence of coal gas (containing carbon monoxide) provided the flasks were kept in motion during the incubation. When the flasks were stationary however less sugar disappeared from the blood that was in contact with coal gas. Thus:

Original percentage of dextrose in blood.....	0.128
Gram dextrose disappeared in 3 hours from 100 grams blood in presence of air	
A. On shaker.....	0.096
B. Stationary.....	0.102
Gram dextrose disappeared in 3 hours from 100 grams blood in presence of coal gas	
A. On shaker.....	0.102
B. Stationary.....	0.074

The relationship between glycolysis and the concentration and source of dextrose in the blood.

One of the main objects of the present research was to see whether any evidence could be obtained indicating that the animal body does not utilize all varieties of dextrose with the same facility. It is a well established fact that the two varieties of dextrose α and β do not exhibit the same behavior toward certain enzymes. It becomes a possibility therefore that the dextrose naturally present in blood which is produced by the enzymic hydrolysis of glycogen differs from commercial dextrose in the relative proportion of the two above varieties which it contains. We are not in possession of any facts that would justify us in concluding that there is really any difference between "commercial" and glycogen dextrose, but we have assumed that if such a difference should exist it might be indicated in the rate at which the two sugars disappear during glycolysis. It is of interest in this connection to note that Stoklasa¹⁵ has claimed that hexoses are "fermented" by alcohol-ether preparations of pancreas juice

¹⁵ Stoklasa: *Zeitschr. f. physiol. Chem.*, lxi, p. 35, 1909.

only when they have been prepared by enzyme hydrolysis. In order to study these questions we have made observations on:

1. Glycolysis in blood containing varying quantities of commercial dextrose.

2. Glycolysis in blood containing varying quantities of glycogen dextrose.

The glycogen dextrose was either prepared *in vitro* by incubating serum with glycogen or it was produced in the intact animal by inducing hyperglycaemia by means of one of the usual methods and then bleeding the animal.

1. The effect of adding commercial dextrose.

In most of the experiments varying quantities of a sterile dextrose solution were added to fresh defibrinated blood, the rate of sugar disappearance being then determined in the original blood and in that containing the dextrose. The results are shown in Table VII.

The most important conclusion which may be drawn from these results is that the addition of dextrose to blood does not as a rule increase the amount of this substance which is destroyed when the blood is incubated for a given time. In other words, the absolute amount of dextrose which disappears is independent of the original concentration of this substance. This fact indicates that the glycolytic power of drawn blood must be very feeble, so feeble indeed that in a given time it can no more than deal with the normal amount of sugar present.

Although the rate of glycolysis cannot be increased by adding dextrose, yet if the incubation be prolonged, amounts of dextrose that are considerably in excess of the normal may ultimately be destroyed (Exp. No. 21). Instead of increasing the glycolysis, the addition of large quantities may slightly depress it, as is shown in case of experiment 16, 22 and X of Table VII. This observation raises the question as to the propriety of adding dextrose to the blood, or other nutritive fluid, employed for perfusing the heart, or other tissues, in order to study sugar utilization. By such additions no other advantage can be gained than that less fluid will have to be taken for analysis while, on the other hand, apart from the possible depression in glycolysis in

TABLE VII.

NO. OF EXP.	TIME OF INCUBATION	BLOOD ALONE		BLOOD + DEXTROSE	
		Per cent dextrose in blood at start	Gms. dextrose disappeared from 100 gms. blood	Per cent dextrose in blood at start	Gms. dextrose disappeared from 100 gms. blood
16	40 min.	0.176	0.025	0.524	0.014
	2 hrs. 10 min.		0.050		0.051
	4 hrs. 10 min.		0.104		0.066
20	1 hr.	0.230	0.025	0.465	
	3 hrs.		0.045		0.070
	5 hrs.		0.094		0.116
21	1 hr.	0.043 (after 24 hrs.' in- cubation.)		0.329	0.039
	3 hrs.				0.054
	4 hrs.				0.075
	20 hrs.				0.257
22	2½ hrs.	0.106	0.047 0.106(?)	A. 0.436	0.031
	4½ hrs.				0.116
	2½ hrs.			B. 0.840	0.087
	4½ hrs.				0.093
	4½ hrs.			C. 1.139	0.093
24	2 hrs.	0.173	0.029	A. 0.329	0.032
	2 hrs.			B. 0.475	0.060
	4 hrs.			C. 0.973	0.072
X	2 hrs.	0.150	0.124	0.351	0.031
	5 hrs.				0.111
26	4 hrs.	none (incu- bated 48 hrs.)		A. 0.351	0.056
				B. 0.665	
	4 hrs.				0.072

the blood itself, the risk is incurred that the excess of dextrose exercises a depressing influence on tissue glycolysis, or it may encourage a deposition of dextrose in a condensed form within the tissues.

Before proceeding to discuss the bearing of these facts on the possible importance of blood glycolysis in the utilization of carbohydrate in the animal body we shall consider the influence produced on glycolysis by adding glycogen dextrose to blood.

In one observation 15 cc. of sterile serum were incubated with 0.1 gram of glycogen for one hour by which time, as control experiments showed, hydrolysis was complete. The resulting serum was then shaken with 15 cc. of centrifuged corpuscles, the percentage of haemoglobin in the mixture being 100. Three quantities of 10 cc. each were then incubated and gave 0.376 per cent dextrose to start, 0.357 after two hours' incubation and 0.332 per cent after four hours, or 0.019 gram dextrose disappeared from 100 grams of blood in two hours and 0.044 in four hours. The above figures, in a control observation with the same serum and corpuscle mixture but containing added commercial dextrose were: 0.474 (start), 0.441 (2 hours), 0.430 (4 hours) or 0.033 gram dextrose disappeared in two hours and 0.044 in four hours. The glycogen dextrose was therefore destroyed at the same rate as commercial dextrose.

However carefully such experiments might be controlled, the objection could always be raised that the manipulations involved in them had altered the glycolytic power of the corpuscles. Experiments were therefore undertaken in which glycolysis was studied in blood removed from hyperglycaemic animals. The hyperglycaemia was induced in some cases by stimulation of the great splanchnic nerve and in others, by curare or adrenalin. In the splanchnic nerve experiments the blood was usually removed after ten minutes' stimulation so that the hyperglycaemia was of slight degree. In the case of the asphyxial and adrenalin experiments it was removed much later so that marked hyperglycaemia existed. In all the experiments before inducing the hyperglycaemia some blood was removed in order that the normal rate of glycolysis might be compared with that occurring in hyperglycaemic blood. The results are shown in Table VIII.

It is very definitely shown, especially by the adrenalin experi-

TABLE VIII.

EXPT. NO.	TIME OF INCUBATION	NORMAL BLOOD			DIABETIC BLOOD				REMARKS
		Per cent dextrose to start	Dextrose disappeared from 100 gms. blood per minute		Method used for producing hyperglycaemia	Per cent dextrose to start	Dextrose disappeared from 100 gms. blood		
			grams	grams			grams	grams	
13	2 hrs. 4 hrs.	0.126	0.022 0.081	0.00018 0.00034	Stim. splanchnic nerve for 10 min.	0.148	0.020 0.089	0.00022 0.00037	Starved dog. Vena cava blood.
14	4 hr. 4½ hrs.	0.172	0.013 0.076	0.00013 0.00028	Stim. splanchnic nerve for 12 min.	0.205	0.009 0.070	0.0003 0.00029	Dog fed sugar. Femoral artery blood.
16	40 min. 4 hrs.	0.176*	0.025 0.104	0.0006 0.00013	Stim. splanchnic nerve for 30 min.	0.200†	0.017 0.062	0.00042 0.00025	Dog fed sugar.
27	2 hrs. 5 hrs.	0.120	0.037 0.081	0.0003 0.00027	Curare injected but respiratory movements persisted.	0.161	0.014		Femoral artery blood.
29	2 hrs. 4 hrs.	0.088	0.051 0.088†	0.0001 0.00032	Adrenalin (10 cc. 1-1000) injections for 1½ hrs.	0.451	0.058 0.078	0.00041 0.00032	Dog fed sugar.
30	30 min. 1 hr. 2 hrs. 4 hrs.	0.223§	0.022 0.073	0.00037 0.00030	Adrenalin injections for 1 hr. 20 min.	0.502	0.010 0.015 0.077	0.00063 0.00034 0.00030	

* Vena cava blood.
† Femoral artery blood.
‡ I.e., all sugar disappeared.
§ Blood very venous.

* Vena cava blood.

† Femoral artery blood.

‡ i.e., all sugar disappeared.

§ Blood very venous.

ments, that even a very great increase in the natural (glycogen) dextrose of the blood does not cause any more than the usual amount of this substance to be destroyed during *in vitro* incubation. There is therefore no apparent difference between the two kinds of dextrose in so far as their destructibility by means of blood is concerned. The glycolytic power of the blood for both commercial and for glycogen dextrose is strictly limited nor does it become appreciably altered in at least two of the varieties of hyperglycaemia (splanchnic stimulation and adrenalin).

From these as well as the foregoing results it is plain that the *glycolysis which we study in blood in vitro does not bear any important relationship to the glycolysis which occurs in the intact animal*. The amount of sugar which disappears from blood *in vitro* is by far too small to account for more than a minute fraction of that which disappears in the body.

Thus, in eviscerated dogs we have found¹⁷ that from 0.83 mgm. to 4.46 mgm. dextrose disappear from 100 grams of blood per minute. Whereas these values for blood *in vitro*, as the above observations show, only range between 0.03 and 0.06 mgm., even during the early stages of the process when it is most rapid. Similar calculation of the results given in the other tables yields corresponding values. These differences between the glycolysis of blood and intact tissues cannot be explained as due to the ready destructibility of the glycolytic agent outside the body for, if this were so, we should expect the glycolytic power to fall off rapidly after the blood is drawn, which however we have seen is by no means the case.

The absence of glycolytic power in the serum and the other facts which make it extremely probable that dextrose must be absorbed into the corpuscles before it can be destroyed further indicate the unimportance of blood glycolysis in the intact animal. Nor does it appear that the intracorpuseular glycolysis can even be made use of to get rid of excessive amounts of circulating dextrose, and thus prevent hyperglycaemia, for we have seen that the process does not become more active under such conditions. Furthermore, the practical constancy in the glycolytic power of the blood before and after an animal is rendered hyperglycaemic

¹⁷ Macleod and Pearce: *Amer. Journ. of Physiol.*, xxxii, p. 184, 1913.

shows that alterations in this process can play no rôle in the cause of the hyperglycaemia.

The supposed existence of "sucre virtuel" in freshly drawn blood.

Lépine and Barral¹⁸ have claimed that the concentration of actual sugar may be greater in blood that has stood for from fifteen minutes to an hour at body temperature outside the body, than in freshly drawn blood. So far as we are aware, no subsequent worker has directly tested this claim.

TABLE IX.

NO. OF EXP.	PER CENT SUGAR IN BLOOD IMMEDIATELY AFTER COLLECTING	PER CENT SUGAR IN SAME BLOOD AFTER STANDING	TIME OF STANDING	REMARKS
			min.	
5	0.107	0.101		This blood stood for some time in ice-cold water before precipitating.
6	{ 0.131 0.135	0.129	15	
7	{ 0.154 0.154	0.129	40	
11	0.207	0.202	20	
13	0.145	0.136	20	From femoral artery.
	0.161	0.148	20	From vena cava.
14	0.172	0.159	30	From femoral artery.
	0.205	0.176	30	From vena cava.
14a	0.205	0.196	30	From femoral artery.

In order to do so, we have compared the sugar in blood collected in ice-cold water and then immediately precipitated with colloidal iron, with that of blood collected either immediately before or afterwards (practically simultaneous) but allowed to stand before precipitation in the incubator for varying periods up to one hour. The results, which are collected in Table IX, show that there was always less sugar in the incubated blood up to 30 minutes after removal. That the decrease becomes more marked after longer periods has already been shown in the previous tables.

¹⁸ Lépine: *Le diabète sucré*, Paris (Felix Alcan), 1909. p. 64.

On account of the absence of any evidence for the supposed increase in sugar, we have in most of our later experiments collected the control blood in water and precipitated it immediately.

CONCLUSIONS.

1. Unclotted (hirudin) and defibrinated blood have the same glycolytic power, but potassium oxalate, in concentrations of one per thousand and over, has a depressing action.

2. The rate of glycolysis varies from time to time in the defibrinated blood of the same animal. It therefore varies also in the blood of different animals of the same species.

3. On an average, about one-half of the original amount of dextrose disappears in two and one-half hours from blood kept outside the body at 40°C.

4. Glycolysis is a function of the corpuscles and is absent in the serum. It disappears from the corpuscles after frequent washing with isotonic saline.

5. The addition of dextrose to blood does not materially increase the extent of the glycolysis occurring in a given time. In higher concentrations it may indeed depress the process.

6. The source of the dextrose, *i.e.*, whether chemical or derived from glycogen by the action of glycogenase, bears no relationship to the rate of glycolysis. Glycolysis proceeds at the same rate in normal as in "diabetic" blood.

7. Even under the most favorable circumstances the quantity of dextrose which the blood can destroy is only a small fraction of that which disappears in the same time in the intact animal. The glycolysis which occurs in blood is most probably of no importance in carbohydrate metabolism.

8. No evidence could be obtained of an increase of sugar as a result of allowing freshly drawn blood to stand at body temperature for varying periods of time up to one hour.

RESEARCHES ON PURINES. XII.¹

ON 2-OXY-6-METHYL-9-ETHYLPURINE, 2-OXY-6,8-DIMETHYL-9-ETHYLPURINE, 2-OXY-6-METHYL-8-THIO-9-ETHYLPURINE, 2-OXY-6-METHYL-9-ETHYLPURINE-8-THIOGLYCOLLIC ACID, AND 2-METHYLMERCAPTO-6-OXY-8-THIOPURINE.

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In the eleventh paper of this series we stated that 2-oxy-4-methyl-5-amino-6-ethyl-aminopyrimidine (II)² reacted readily, when heated with urea, to form 2,8-dioxy-6-methyl-9-ethylpurine (VI).³ We find that this pyrimidine also reacts smoothly with other reagents which are commonly used for the preparation of purines from orthodiaminopyrimidines.

Thus, when the formyl derivative of 2-oxy-4-methyl-5-amino-6-ethylamino pyrimidine is heated, water is liberated and 2-oxy-6-methyl-9-ethylpurine (I) is formed. When the corresponding acetyl compound was heated we obtained 2-oxy-6,8-dimethyl-9-ethylpurine (III). The yields in both cases were excellent. This diaminopyrimidine also reacts normally when heated with thiourea and forms 2-oxy-6-methyl-8-thio-9-ethylpurine (IV). The yield was 61 per cent of the calculated. The thiopurine, thus obtained, reacted with monochloroacetic acid and gave 2-oxy-6-methyl-9-ethylpurine-8-thioglycollic acid (V). This compound is stable in hot water but when boiled with concentrated hydrochloric acid it is hydrolyzed to 2,8-dioxy-6-methyl-9-ethylpurine (VI). The thioglycollic acid derivative forms a crystalline ammonium salt. We have also investigated the action of thiophosgene on 2-methyl-

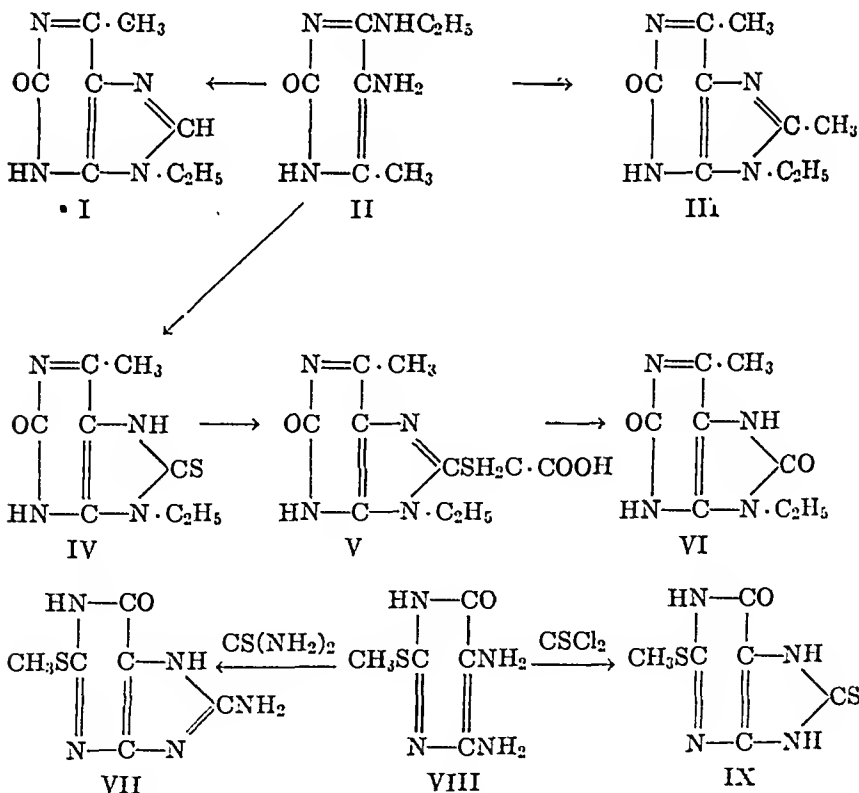
¹ Johns and Baumann: this *Journal*, xv, p. 119, 1913.

² Johns and Baumann: *ibid*, xv, p. 123, 1913.

³ Johns and Baumann: *ibid*, xv, p. 124, 1913.

mercapto-4,5-diamino-6-oxypyrimidine (VIII)⁴ and find that 2-methylmercapto-6-oxy-8-thiopurine (IX) is formed. The yield was low, about 44 per cent of the calculated, but a suitable solvent for the diaminopyrimidine was not found and the reaction was carried out in the presence of water which decomposed the thiophosgene to a considerable extent. This formation of 2-methylmercapto-6-oxy-8-thiopurine is of interest since we found previously that when thiourea and 2-methylmercapto-4,5-diamino-6-oxy-pyrimidine were heated together the resulting compound was 2-methylmercapto-6-oxy-8-aminopurine (VII)⁵ instead of the expected 2-methylmercapto-6-oxy-8-thiopurine (IX).

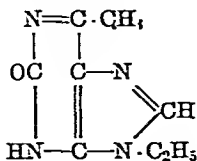
These researches will be continued.



⁴ Johnson, Johns and Heyl: *Amer. Chem. Journ.*, xxxvi, p. 172, 1906.
 Johns and Baumann: this *Journal*, xiv, p. 385, 1913.

⁵ Johns and Baumann: this *Journal*, xiv, p. 387, 1913.

EXPERIMENTAL PART.

2-Oxy-6-methyl-9-ethylpurine.

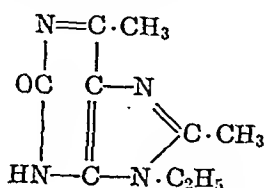
Three grams of 2-oxy-4-methyl-5-amino-6-ethyl-aminopyrimidine⁶ were dissolved in 15 cc. of 85 per cent formic acid. Solution took place at room temperature and considerable heat was evolved. This solution was then boiled gently under a return condenser for fifteen minutes and was finally evaporated to dryness on a steam bath. The residue was heated in an air bath at 170°-180°C. for an hour. The reaction product was dissolved in hot water and clarified once with blood coal. This treatment left a colorless solution which was concentrated to a small volume and allowed to cool slowly. 2-Oxy-6-methyl-9-ethylpurine crystallized out in needles which formed a network with a silky appearance. A second crop of crystals was obtained by concentrating the mother liquor. The yield was nearly quantitative. This purine did not contain water of crystallization after it had been dried over sulphuric acid for three days. It began to melt with partial decomposition at about 256°C. and at 275°C. all had melted to a dark oil. It was very soluble in hot water or alcohol as well as in cold dilute acids and alkalis. It did not dissolve in ether and was but slightly soluble in benzene. An aqueous solution of the purine did not give a precipitate with barium chloride or picric acid but gave a white precipitate with mercuric chloride. This was soluble in hot water and separated again on cooling. The purine gave a white gelatinous precipitate with ammoniacal silver. Nitric acid oxidized the purine readily and on evaporation a yellow crust remained. This turned red when it was moistened with a drop of alkali and dried on the steam bath.

⁶ Johns and Baumann: this *Journal*, xv, p. 123, 1913.

0.1448 gram of substance gave 0.2871 gram of CO_2 and 0.0708 gram of H_2O .

	Calculated for $\text{C}_8\text{H}_{10}\text{ON}_4$:	Found:
C.....	53.93	54.07
H.....	5.62	5.46
N.....	31.46	31.51

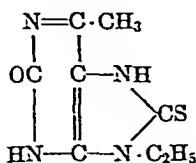
2-Oxy-6,8-dimethyl-9-ethylpurine.



Three grams of 2-oxy-4-methyl-5-amino-6-ethylaminopyrimidine were dissolved in 15 cc. of acetic anhydride and the solution was boiled for five minutes after which it was evaporated to dryness on a steam bath. The residue was then heated in an air bath at 180°C . for an hour. It was then dissolved in water and the solution was decolorized with blood coal. On concentrating to a small volume and cooling slowly a network of silky needles was obtained. These needles were found to be anhydrous after drying over sulphuric acid for three days. The yield was almost theoretical. The crystals began to shrink at about 230°C . and melted to a dark oil at about 265°C . This purine was very soluble in hot water or alcohol and also in cold dilute acids and alkalis. It was not soluble in ether and only slightly soluble in benzene. Its aqueous solution did not give a precipitate with barium chloride or picric acid but gave white precipitates with solutions of mercuric chloride and ammoniacal silver. Nitric acid attacked the purine readily and when the resulting solution was dried a yellow residue remained. This turned red when moistened with ammonia.

0.2098 gram of substance gave 0.4313 gram of CO_2 and 0.1176 gram of H_2O .

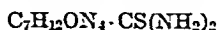
	Calculated for $\text{C}_{10}\text{H}_{12}\text{ON}_4$:	Found:
C.....	56.25	56.07
H.....	6.25	6.23
N.....	29.17	29.26

2-Oxy-6-methyl-8-thio-9-ethylpurine.

Six grams of 2-oxy-4-methyl-5-amino-6-ethylaminopyrimidine and an equal weight of thiourea were pulverized together and the mixture was heated for an hour at 175° to 185°C. in an oil bath. As the thiourea melted the mass darkened, frothing occurred and in about a half hour the whole mass solidified. The reaction product was dissolved in hot dilute ammonia and after clarifying with blood coal the solution was acidified with acetic acid. The purine was thrown down at once from the hot solution. It was purified by dissolving in hot dilute ammonia and precipitating with acetic acid. After drying at 100°C. the yield was 4.2 grams or 61 per cent of the calculated weight. The substance, thus obtained, decomposed at 295° to 300°C. It was difficultly soluble in hot water and on cooling the solution slowly it crystallized in sheaves. It was slightly soluble in hot alcohol but did not dissolve in benzene or ether. It dissolved readily in dilute acids or alkalies. Its aqueous solution did not give a precipitate with barium chloride or picric acid. It formed insoluble compounds with mercuric chloride and ammoniacal silver solutions. Nitric acid oxidized it readily, leaving a yellow residue on evaporation. This turned red where moistened with ammonia.

	Calculated for $\text{C}_7\text{H}_{10}\text{ON}_4\text{S}$:	Found:	
		I	II
N.....	26.67	26.92	26.79

The thiourea addition product of 2-oxy-4-methyl-5-amino-6-ethylaminopyrimidine.



Six-tenths gram of 2-oxy-4-methyl-5-amino-6-ethylaminopyrimidine were dissolved in a little hot water and an equal weight of thiourea was added. The thiourea dissolved, reaction took place and a solid substance separated rapidly. After cooling, the

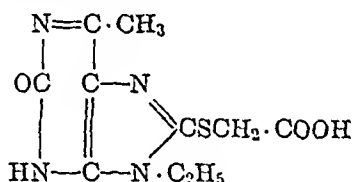
solid was filtered off and washed with a little cold water. It was rather soluble in hot water and sparingly soluble in cold water and but slightly soluble in alcohol. The crystals melted to a dark oil at 204°-206°C.

	Calculated for $C_8H_{10}ON_4S$:	Found:
N.....	34.43	34.38

When the above compound was heated at 175°-185°C. ammonia was evolved, frothing and darkening took place and a solid residue was formed. When this product was dissolved in dilute ammonia, the solution clarified with blood coal and acidified with acetic acid, a precipitate of 2-oxy-6-methyl-8-thio-9-ethylpurine resulted.

	Calculated for $C_8H_{10}ON_4S$:	Found:
N.....	26.67	26.79

2-Oxy-6-methyl-9-ethylpurine-8-thioglycollic acid.



Two grams of 2-oxy-6-methyl-8-thio-9-ethylpurine were suspended in 150 cc. of water containing 10 grams of monochloroacetic acid and the mixture was boiled under a return condenser until the purine had dissolved. This required about an hour. After filtering to remove a slight turbidity the solution was cooled, whereupon a bulky mass of needles crystallized out. A second crop was obtained by concentrating the mother liquor. The yield was 1.7 grams or 65 per cent of the theoretical amount. The thioglycollic acid derivative was readily soluble in hot and moderately soluble in cold water. It was slightly soluble in hot alcohol and not soluble in benzene. It was moderately soluble in hot glacial acetic acid and dissolved readily in dilute mineral acids or alkalis. It dissolved in hot dilute ammonia and on cooling the solution hair-like crystals were obtained. It began to darken at 270°C. and decomposed gradually at higher temperatures.

	Calculated for $C_{10}H_{12}O_3N_4S$:	Found:
N.....	20.90	20.99

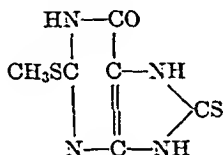
Hydrolysis of 2-oxy-6-methyl-9-ethylpurine-8-thioglycollic acid.

When this thioglycollic acid derivative was heated with concentrated hydrochloric acid under a reflux condenser for two hours hydrolysis occurred. The resulting solution was evaporated to dryness and the residue was dissolved in ammonia and this solution was acidified with acetic acid. The compound thus obtained crystallized in sheaves and exhibited all the properties of 2,8-dioxy-6-methyl-9-ethylpurine. It was free from sulphur and did not melt or decompose at 320°C.

Calculated for
C₈H₁₃O₂N₄:

Found:

N..... 28.87 28.93

2-Methylmercapto-6-oxy-8-thiopurine.

Five grams of 2-methylmercapto-6-oxy-4,5-diaminopyrimidine⁷ were dissolved in boiling water and the solution was cooled rapidly in order to obtain small crystals. Fifteen grams of thiophosgene were added gradually with thorough shaking. This operation was carried out in a glass stoppered bottle. The solvent became blue and a granular substance separated. This was filtered off, dissolved in hot dilute ammonia and reprecipitated with acetic acid. It crystallized in small globules which were soluble in about 200 parts of boiling water and almost insoluble in cold water. They did not dissolve in ether and were but slightly soluble in hot alcohol or glacial acetic acid. They began to decompose at about 275°C. They gave the murexide test. The yield was low since a suitable solvent for the diaminopyrimidine was not found and water decomposed the thiophosgene. The amount obtained from 5 grams of the diaminopyrimidine was only 1.5 grams. However, 1.6 grams of the diaminopyrimidine were recovered by evaporating the mother liquor thus making the yield of thiopurine about 44 per cent of the theoretical amount.

Calculated for
C₈H₈O₂N₄S:

Found:

N..... 26.17 26.13 26.16

⁷ Johns and Baumann: this *Journal*, xiv, p. 385, 1913.

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